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DEVELOPMENT OF THE PARTS OF SEA URCHIN EGGS SEPARATED BY CENTRIFUGAL FORCE

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With rapid centrifuging in the proper medium, the eggs of *Sphærechinus granularis*, *Parechinus* (*Echinus*) *microtuberculatus*, *Paracentrotus* (*Strongylocentrotus*) *lividus*, and *Arbacia pustulosa*, the four commonly occurring sea urchins at Naples, and *Tripneustes* (*Hipponoë*) *esculenta* from Bermuda can be broken apart into halves and some of these halves into quarters. The results confirm and complement those previously obtained with the eggs of the Woods Hole sea urchin, *Arbacia punctulata*, which have recently been published (1932). The speed ordinarily used was about 9,000 revolutions per minute (7 cm. radius), and the time necessary to break the eggs apart was from three minutes for *Arbacia pustulosa* to thirty minutes for *Paracentrotus lividus*. The medium used was about half sea water and half 1.1 molal sucrose solution (376 grams sucrose in 1 liter of distilled water) which is isotonic with the eggs; the Δ of the Naples sea water is -2.2 to -2.3 whereas that of the Woods Hole sea water is -1.81; this is also of practically the same density as the eggs themselves, so that they remain suspended in the solution during centrifuging; in the case of the heavier eggs of *Arbacia pustulosa*, a larger proportion of sugar solution was used (3 parts 1.1 molal sucrose to 1 part sea water).

SPHÆRECHINUS GRANULARIS

Normal Egg

The egg of *Sphærechinus granularis* is colorless or slightly yellowish and very granular. It measures 94–102 μ in diameter, averaging 98 μ; the eggs of any one batch are fairly constant in size but different batches vary. The nucleus measures 13 μ in diameter, increasing to 18 μ before it breaks down prior to cleavage. The eggs are usually quite aspherical

[1] I wish to express my sincere thanks to Dr. Reinhard Dohrn, Director of the Stazione Zoologica, for his interest and courtesy during my stay in Naples, and to the Committee of the American Woman's Table for the use of their research room.

when laid and remain so for several hours, but become spherical almost immediately on fertilization. The fertilization membrane is well separated from the egg surface, leaving a perivitelline space of 18–20 μ; the ectoplasmic layer is fairly thick, measuring 3–4 μ. The egg develops comparatively slowly, taking about 1¾ hours for first cleavage at 16° (50 per cent cleaved). It passes through a typical monaster stage (25–45 minutes after fertilization) and streak stage (50–90 minutes). The density of the egg with jelly is about 1.083 (eggs evenly distributed in 1.1 m sucrose 3 parts: sea water 2 parts) and without jelly about 1.081 (eggs float in 1.1 m sucrose 3 parts: sea water 2 parts, sink in 1.1 m sucrose 5 parts: sea water 4 parts).[2]

Centrifuged Egg

The unfertilized eggs break apart fairly easily with centrifugal force, usually in 5 minutes with about 9,000 r.p.m. (7 cm. radius) though some batches require longer and some less. The eggs become elongate, then dumb-bell-shaped (Fig. 1 and Photograph 1), then break into two parts with often a connecting strand between (Fig. 2, Photograph 7). If after centrifuging, the strand is very narrow, the parts remain separate; otherwise the strand gradually becomes thicker and the two parts merge into a single sphere.

The stratification is (1) oil, (2) clear layer in which lies the nucleus (3) whitish, loosely-packed granules merging into (4) yellow yolk granules and (5) usually a small clear zone (Fig. 1, Photograph 1). The granules measure approximately in diameter:

Oil	1 μ
White granules	1.5 μ
Yellow yolk granules	1–1.5 μ

[2] The computations for density of the eggs are based on Wendicke's (1916) figures for the salinity of Naples sea water (37.5 parts salt per 1000 cc.) giving a density of 1.0278 at 16°; and the density of 1.1 molal sucrose, found to be about 1.119 at 16°.

Description of Plates

The drawings have been made from living eggs and are magnified about 400 ×. The small circles represent oil drops, the large solid dots (in *Arbacia pustulosa*) red pigment granules, and the stippling represents yolk and other granules, the fine stippling fine granules, and the coarse stippling coarser granules.

Plate I. *Sphærechinus granularis*

1. Stratified whole egg.
2. Whole egg broken into two with a connecting strand between the two parts.
3. White half egg.
4. Granular half egg.
5. White half egg pulling apart.
6. Clear quarter egg.
7. White granular quarter.
8. Granular half egg pulling apart.
9. Upper yolk quarter egg.
10. Lower yolk quarter egg.

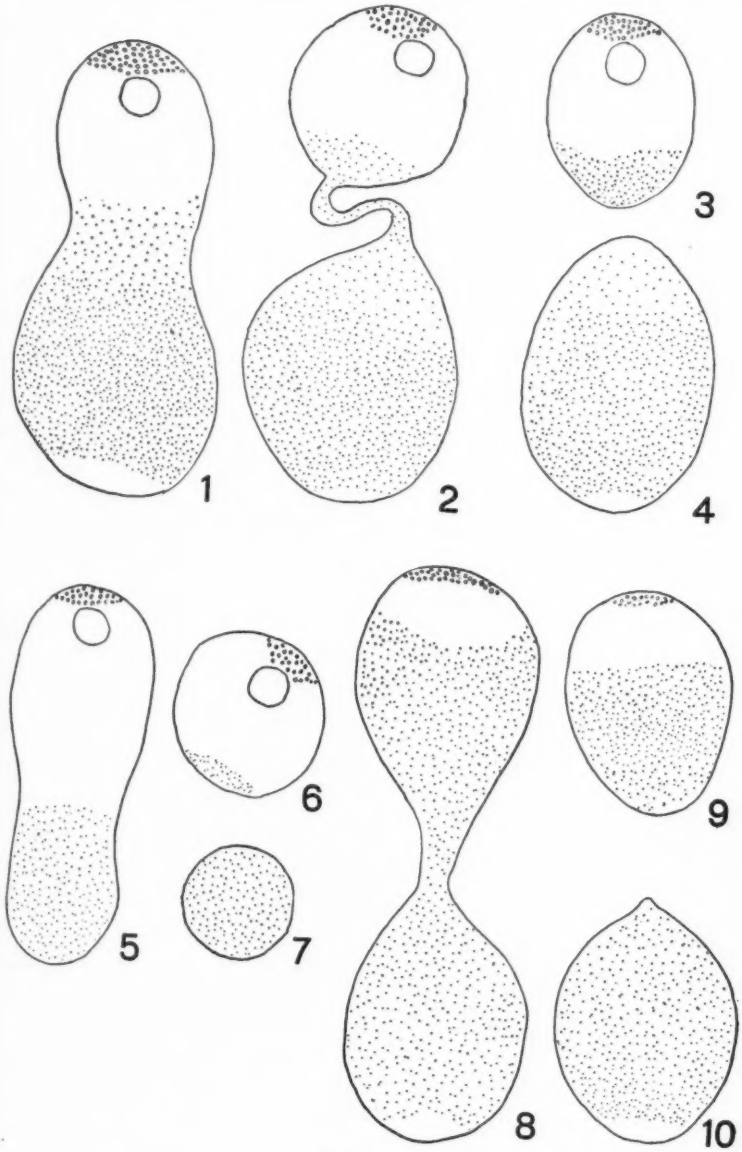
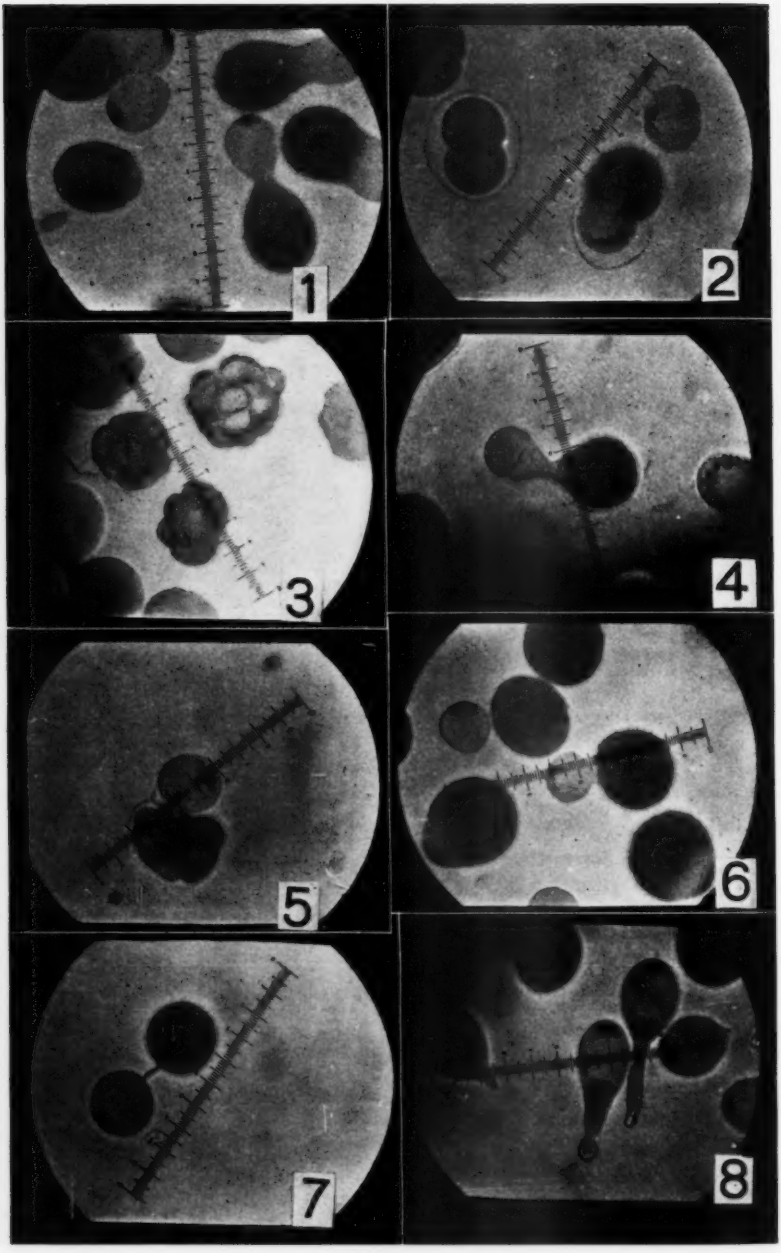


PLATE I



PHOTOGRAPHS 1–8

The white granules stain purple with methyl green like those in the fifth layer of *Arbacia punctulata* (mitochondria?).[3]

Half and Quarter Eggs

The eggs break apart at 9,000 r.p.m. across the whitish granules, giving two parts, one smaller nucleated half egg containing oil, clear layer and whitish granules (mitochondria?) (Fig. 3 and Photograph 1) and the other half egg, larger, non-nucleate, containing a few whitish granules (mitochondria?) but mostly yellow yolk granules with usually a clear layer at the heavier pole; there is usually also a clear layer at the lighter pole from which granules have been thrown down (Fig. 4 and Photograph 1). In the centrifuge tube, there are not three well-separated layers as in *Arbacia punctulata*, but the layers are contiguous, a whitish layer lying just above a yellowish layer which consists both of dumb-bell-shaped whole eggs above and granular half eggs below. When the eggs are first broken apart, the half eggs are usually not spherical but somewhat elongate, especially the granular half (Photograph 1). In some batches of eggs, without further centrifuging, some of both the clear halves and the yolk halves again become dumb-bells (Figs. 5, 8) and many break apart into quarter eggs. The white half egg separates into an almost clear quarter egg with oil cap and nucleus and a few whitish granules (Fig. 6), and a smaller quarter egg containing only whitish granules (Fig. 7). The granular half egg divides into two somewhat similar parts but the upper quarter egg has a few oil drops at the lighter pole, a clear layer, a few whitish granules and yolk granules (Fig. 9), and the heavier quarter egg has yolk granules and a small clear zone at the blunt end (Fig. 10); the position of the breaking point is often marked by a slight pointed projection. These

[3] These granules and similar granules in the other eggs which stain with methyl green, stain also with Janus green B, very kindly given me by E. V. Cowdry. They are without doubt mitochondria (Naples, February 1933).

Photographs 1–8. *Sphærechinus granularis*

1. Whole egg at right; two half eggs at left.
2. Two-cell stage; granular half at left, white half above, whole egg below.
3. Late segmentation stage; granular half eggs at left; whole egg above.
4. Development of white portion, no development of granular portion of partially separated whole egg.
5. Development of granular portion, no development of white portion of partially separated whole egg.
6. High speed (about 13,000 r.p.m.); granular half much larger than white half. Both half eggs spherical.
7. Low speed (about 5,000 r.p.m.); two half eggs nearly same size; connecting strand between two half eggs.
8. Low speed, white half eggs with tails.

TABLE I

Size of parts. Diameters in μ; volumes in μ^3 to be multiplied by 10^3.

	Spaerechinus granularis	*Parechinus microtuberculatus*		*Paracentrotus lividus*	*Arbacia pustulosa*	*Tripneustes esculenta*
Speed (7 cm. radius)	9,000 r.p.m.	9,000 r.p.m.	13,000 r.p.m.	9,000 r.p.m.	8,000 r.p.m.	8,000 r.p.m.
Whole Egg						
Diameter	96	102	102	90	77	83
Volume	463.2	555.6	555.6	381.7	239	299.4
Nucleate Half						
Diameter	70	90	96	67	67	77
Volume	179.6	381.7	463.2	157.4	157.4	239
Non-nucleate Half						
Diameter	83	72	58	74	53	47
Volume	299.4	195.3	102.1	212.2	78	54.4
Nucleate Quarter						
Diameter	63				61	
Volume	131.9				118.8	
Non-nucleate Quarter (from nucleate half)						
Diameter	47				38	
Volume	54.4				28.7	
Upper Yolk Quarter						
Diameter	66		48		42	
Volume	150.5		57.9		40.3	
Lower Yolk Quarter						
Diameter	66		45		42	
Volume	150.5		47.7		40.3	

two yolk quarters are of approximately the same size. The smallest quarter egg is the white non-nucleate granular quarter from the white half, and this measures about one-ninth (in one batch one-thirteenth) the volume of the whole egg.

The measurements of the halves and quarters broken apart at 9,000 r.p.m. are given in Table I. There is a slight variation in the relative size of parts in different batches of egg. The data given are for one typical batch.

Relation of Speed to Size of Half Eggs

The relative size of the two half eggs is fairly constant for a constant centrifugal speed. The size of the parts, however, is different for different speeds. With high speed, about 13,000 r.p.m. (7 cm. radius 50 seconds), the granular sphere is much larger than the white sphere (Photograph 6); with low speed, about 5,000 r.p.m. (7 cm. radius 1 hour) the granular half is relatively smaller so that the two halves are almost equal in size (Photograph 7). There is also a difference in the way they break at different speeds. With high speed, the two parts break off as spheres, whereas with low speed there is an elongation of the parts, and often a thick strand of tissue between the two which is left as a tail on one or both parts when separated (Photograph 8). The sizes of parts for one batch of eggs at different speeds are given in Table II.

Development

All of the half and quarter eggs, as well as the elongate and dumb-bell-shaped whole eggs can be fertilized. They throw off fertilization membranes, well separated from the surface as in the normal egg. The membrane follows the contour of the surface even along the strand connecting the two half eggs or along the stalk that is sometimes left after breaking apart (Photograph 8).

In the nucleate half eggs, the stages following fertilization are as in the normal eggs, except for absence of astral rays where granules are absent; and practically as described for the white half eggs of *Arbacia punctulata* (E. B. Harvey, 1932). The first cleavage of a white half egg is shown in Photograph 2; other cleavages follow quite regularly. The blastulæ resulting from the white half egg are at first vacuolated and many remain thus, but many invaginate a day or two later than the normal eggs, form a rudimentary skeleton and pigment spots, but remain almost spherical even for ten days without developing arms. I have never raised any perfectly normal plutei from these half eggs, and they are not very viable after the blastula stage.

TABLE II

Speed and size of parts. Diameters in μ; volumes in μ^3 to be multiplied by 10^3.

Speed (7 cm. radius)			5,000 r.p.m.		8,000 r.p.m.		13,000 r.p.m.	
	Diam.	Vol.	Diam.	Vol.	Diam.	Vol.	Diam.	Vol.
Sphaerechinus granularis egg	102	555.6						
Nucleate half			80	268.0	71	187.4	58	102.1
Non-nucleate half			83	299.4	88	356.8	96	463.2
Parechinus microtuberculatus egg	99	508.0						
Nucleate half			86	332.9	90	381.7	91	394.6
Non-nucleate half			70	179.6	65	143.8	61	118.8
Arbacia pustulosa egg	81	278.3						
Nucleate half			64	137.2	59	107.5	54	82.4
Non-nucleate half			65	143.8	69	172.0	72	195.4
			7,000 r.p.m.					
Paracentrotus lividus egg	90	381.7						
Nucleate half			69	172.0	67	157.4	58	102.1
Non-nucleate half			72	195.3	74	212.2	80	268.0

When the enucleate (merogonic or androgenetic) half eggs are fertilized, the sperm aster develops in the granules in about 10 minutes, a nucleus forms and increases in size as the aster increases, giving a typical monaster stage. This is followed by a " streak " stage, the streak probably being due to the division of the sperm centrosome. The streak fades out, the nucleus enlarges and disappears, and an amphiaster appears, followed by cell division soon after the controls. In aspherical half eggs, the amphiaster lies near the less granular zone, and the division plane divides the cell unequally across the short axis. In spherical eggs, the first division is along the equator (Photograph 2) and this is followed by regular divisions; Photograph 3 shows a late cleavage stage of two of these half eggs and a normal egg. At the fourth division colorless micromeres can sometimes be distinguished. Perfectly normal blastulæ result and become free-swimming about an hour after the controls. These invaginate and acquire a skeleton and pigment about a day later than the controls. Many of the larvæ remain with a well-developed gut, rudimentary skeleton, and pigment spots and do not obtain the typical arms of the pluteus. Some of the larvæ, however, form a complete skeleton and acquire arms and become exactly like the normal plutei except that they are smaller. Some of these small plutei have been kept for 20 days. The androgenetic larvæ are more normal and viable than the larvæ from the nucleated half eggs.

The yellow granular quarter eggs from the granular half eggs develop in the same way as described for the granular half eggs and form regular swimming blastulæ.

The white granular quarter eggs from the white half egg also cleave quite regularly and form swimming blastulæ; some plutei were obtained from these with gut, rudimentary skeleton, and pigment spots. This quarter egg is only one-ninth the volume of the original egg and contains no nucleus, oil or yolk granules and yet can give rise to a pluteus, which though not quite normal has all the fundamental parts of the normal pluteus. The clear quarter eggs may divide equally but long after the controls, and do not develop far. In some of these quarters, the nucleus becomes very large, half the diameter of the cell.

When the elongate or dumb-bell-shaped whole eggs are fertilized, the sperm may enter at any point and an entrance cone is formed. The fertilization membrane starts at this point and follows the contour of the egg. Within a few minutes after the membrane is formed, the egg often pulls away from one or both ends of the membrane, becoming less aspherical (Fig. 11). This probably indicates a decrease in viscosity immediately following fertilization. If the constriction of the dumb-bell is wide, the two pronuclei approach and fuse, usually near the

clear end, and the egg divides unequally across the short axis, giving a smaller clear cell and a larger granular cell (Photograph 2), as described for *Arbacia punctulata*. The second division usually comes in at right angles, but sometimes parallel with the first, resulting in a single row of four cells (Fig. 12). By subsequent divisions, a slipper-shaped blastula is formed as in *Arbacia punctulata* (cf. Photograph 17). This gives rise to a normal pluteus.

When the dumb-bell-shaped egg has been broken by the centrifugal force into two half eggs with a thin strand of protoplasm between (Photograph 7), the fertilization membrane forms also over this and usually over both spheres; there is often an appreciable lag (of about 1 minute) in the formation of the membrane over the second sphere. Occasionally the sphere not entered by the sperm does not form a fertilization membrane and does not develop. One or both spheres may receive a sperm. If the sperm enters the nucleate sphere alone, it develops and the granular sphere does not, at least in the cases that I have observed (Photograph 4). If the sperm enters the granular sphere alone, this sphere may develop without the other (Photograph 5) or both spheres may develop (Figs. 13, 14). If both spheres receive a sperm, both usually develop. The two parts develop quite independently, even the first cleavage not being synchronous (Fig. 14). The protoplasmic strand connecting the two half eggs is sometimes broken early in development (Fig. 13). Both parts often develop into blastulæ forming twins swimming around together.

Parechinus (Echinus) microtuberculatus

Normal Egg

The egg of *Parechinus* (*Echinus*) *microtuberculatus* is colorless, not very granular, but quite clear and transparent. Its diameter measures 96–113 μ, averaging 102 μ; the nucleus measures 13 μ. The eggs are quite aspherical when laid and remain so for several hours, but round up immediately on fertilization. The fertilization membrane is well separated from the egg surface, leaving a perivitelline space of 21 μ; the ectoplasmic layer when fully formed measures 2–3 μ. First cleavage takes place in 70 minutes at 16°. The density of the egg with jelly is about 1.074 (sometimes float, sometimes sink in 1.1 m sucrose 1 part: sea water 1 part), and without jelly about 1.072 (float in 1.1 m sucrose 1 part: sea water 1 part).[2]

Plate II. *Sphærechinus granularis*

11. Egg slipped back from fertilization membrane, soon after fertilization.
12. Four-cell stage, second division plane parallel with first.
13. Independent development of two halves of egg partially separated, connecting strand broken.
14. Another egg, later stage, connecting strand still present.

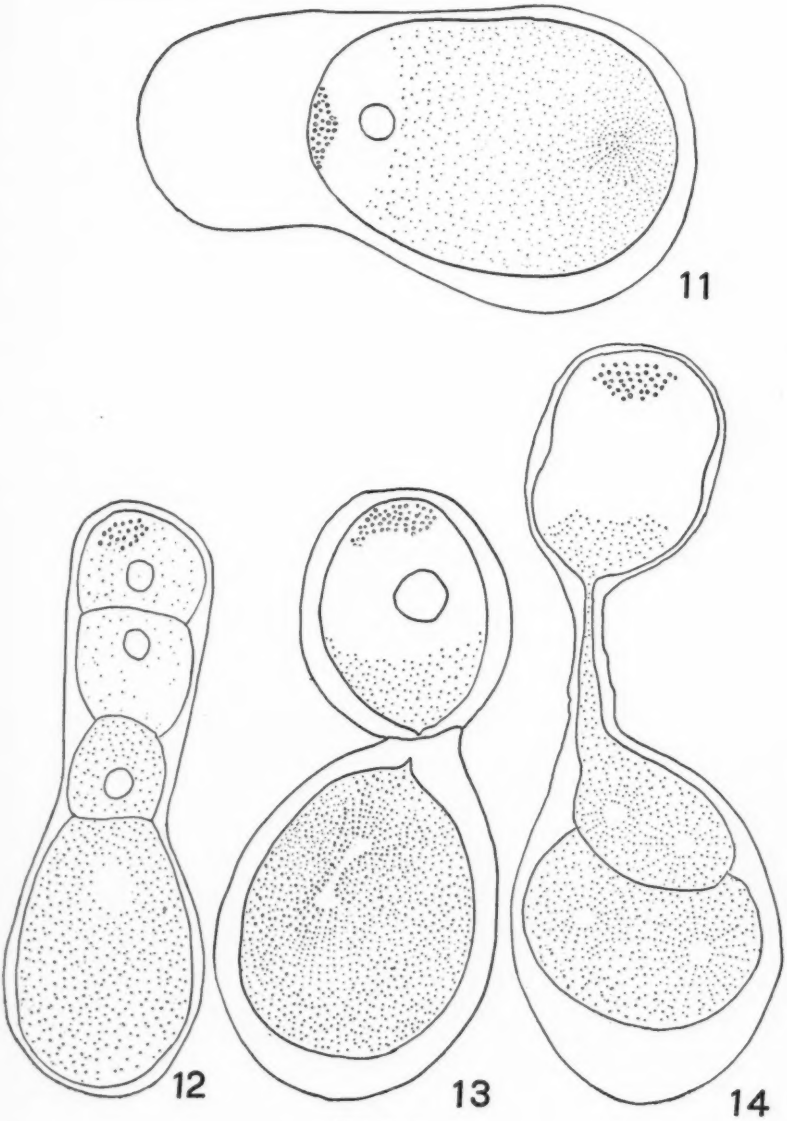
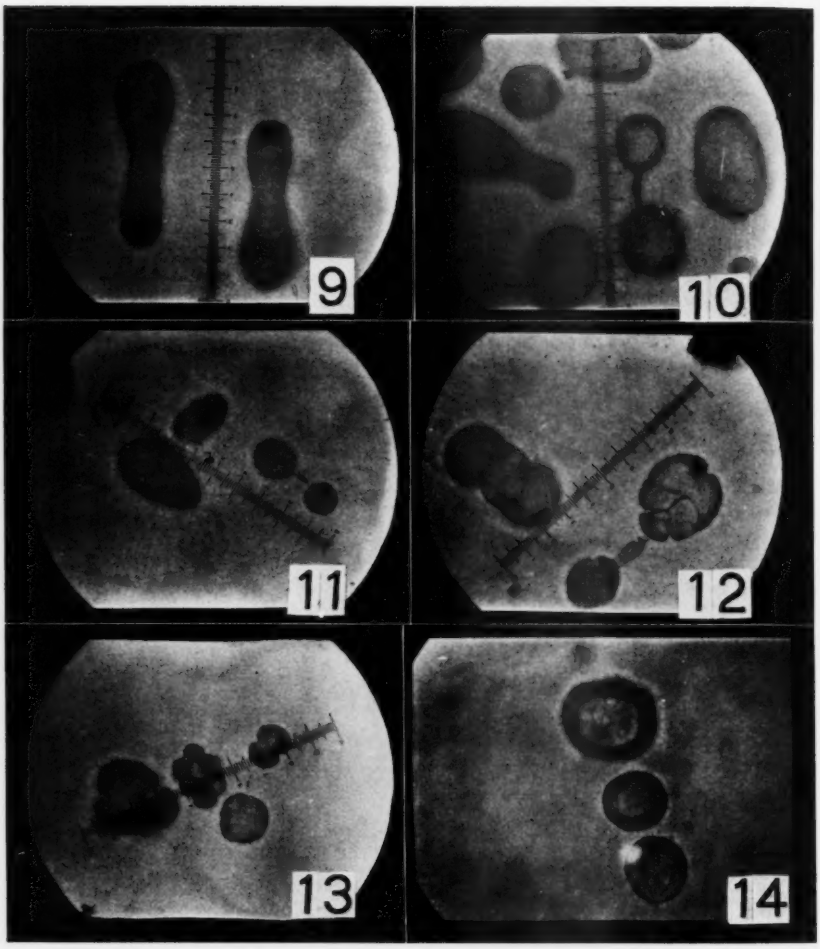


PLATE II



Photographs 9-14. *Parechinus (Echinus) microtuberculatus*

9. Whole egg pulling apart; cap of fine yellow granules at one end.

10. Egg pulled apart with connecting strand.

11. Two half eggs at left; yellow granular half pulled apart into quarter eggs with strand between, at right.

12. Four-cell stage of whole egg at left, white half at right, yellow granular half below, with connecting strand between the two halves.

13. Cleavage of white half at left, granular half in center; two quarter eggs (from yellow granular half) at right connected by a strand.

14. Blastula of whole egg above; of two yellow granular half eggs below.

Centrifuged Egg

When the egg is centrifuged at about 9,000 r.p.m. the stratification is (1) oil (2) coarse white granules at the lighter end in which lies the nucleus (3) almost clear zone (4) very small, closely-packed yellowish granules (Fig. 15, Photograph 9). This egg (and that of *Tripneustes*) differs from the others studied in having granules lighter than the clear zone, which lie under the oil cap, and the nucleus lying among these granules instead of in a clear zone. A similar condition has been described for *Tubifex rivulorum* by Parseval (1922). The relative area of coarse white granules and clear zone differs in different lots owing probably to the amount of packing of the granules. The granules measure approximately:

Oil	0.7 μ
Coarse white granules	2 μ
Fine yellow granules	0.4 μ

The small yellow granules at the heavy end stain purple with methyl green (mitochondria?).

Half and Quarter Eggs

The eggs break apart at about 9,000 r.p.m. usually in 8 minutes with often a connecting strand between the two parts (Photograph 10). The larger sphere contains oil, nucleus, and coarse white granules, and the smaller sphere consists of an almost clear half and a yellowish half composed of the fine yellow granules (mitochondria?). The measurements for a typical lot at 9,000 r.p.m. are given in Table I.

In some batches of eggs the small enucleate halves formed dumbbells and broke into two spheres, some connected with each other by a thin strand of tissue (Photograph 11). The halves and quarters obtained in one batch broken at about 13,000 r.p.m. are given in Table I.

Speed and Size

The size of the two half eggs varies somewhat with the centrifugal speed. In all the other species, the higher speed increases the size of the granular or heavier sphere. In *Parechinus* the heavier sphere decreases in size with high speed, and increases with low speed. In Table II are given the data for one lot of eggs.

Development

All of the half and quarter eggs can be fertilized and throw off well-separated membranes like the whole egg. They all cleave and form swimming blastulæ (Photographs 12, 13, 14); and some dwarf plutei were raised from the enucleate halves. The elongate whole egg when

fertilized often slips away from one or both ends of the membrane, becoming less aspherical as noted for *Sphærechinus*. When the two parts are connected by a strand of tissue, they often develop independently (Photograph 12) and form twin blastulæ. One part may, however, develop and the other part remain undeveloped, just as described for *Sphærechinus*.

Paracentrotus (Strongylocentrotus) lividus

Normal Egg

The egg of *Paracentrotus* (*Strongylocentrotus*) *lividus* is clear and transparent and not very granular. It is colorless except for an orange or reddish band that encircles the egg just below the equator; this is very inconspicuous in some batches of eggs and quite noticeable in others as noted by many other observers. The diameter of the egg is 83–95 μ, averaging 90 μ; the nucleus measures 12 μ, increasing to 16 μ before cleavage. The eggs are not very spherical when laid, but are more so than are those of *Sphærechinus* and *Parechinus*. The fertilization membrane is fairly well separated from the egg, leaving a perivitelline space of 10–12 μ. The ectoplasmic layer is thin, 1–2 μ. First cleavage takes place in 1½ hours at 16°. The density of the egg with jelly is about 1.083 (eggs evenly distributed in 1.1 sucrose 3 parts: sea water 2 parts); without jelly about 1.079 (evenly distributed in 1.1 m sucrose 5 parts: sea water 4 parts).[2]

Centrifuged Egg

This egg is much more difficult to break apart than either *Sphærechinus* or *Parechinus;* it takes about 30 minutes at 9,000 r.p.m. and none of the eggs were broken into quarters. The stratification is (1) oil (2) clear layer in which lies the nucleus (3) coarse granules (4) fine granules (Fig. 16, Photograph 15). The granules measure approximately:

Oil	0.8 μ
Coarse granules	1.5 μ
Fine granules	0.5–1.5 μ

The fine granules stain purple with methyl green (mitochondria?). The orange band is not thrown down in the mature eggs by centrifugal

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Plate III

15. *Parechinus* (*Echinus*) *microtuberculatus*, stratified whole egg.
16. *Paracentrotus* (*Strongylocentrotus*) *lividus*, stratified whole egg.
17. *Paracentrotus*, tripartite egg, all three parts with sperm asters.
18. Same developed into triple blastula.
19. *Arbacia pustulosa*, stratified whole egg.
20. *Tripneustes* (*Hipponoë*) *esculenta*, stratified whole egg.

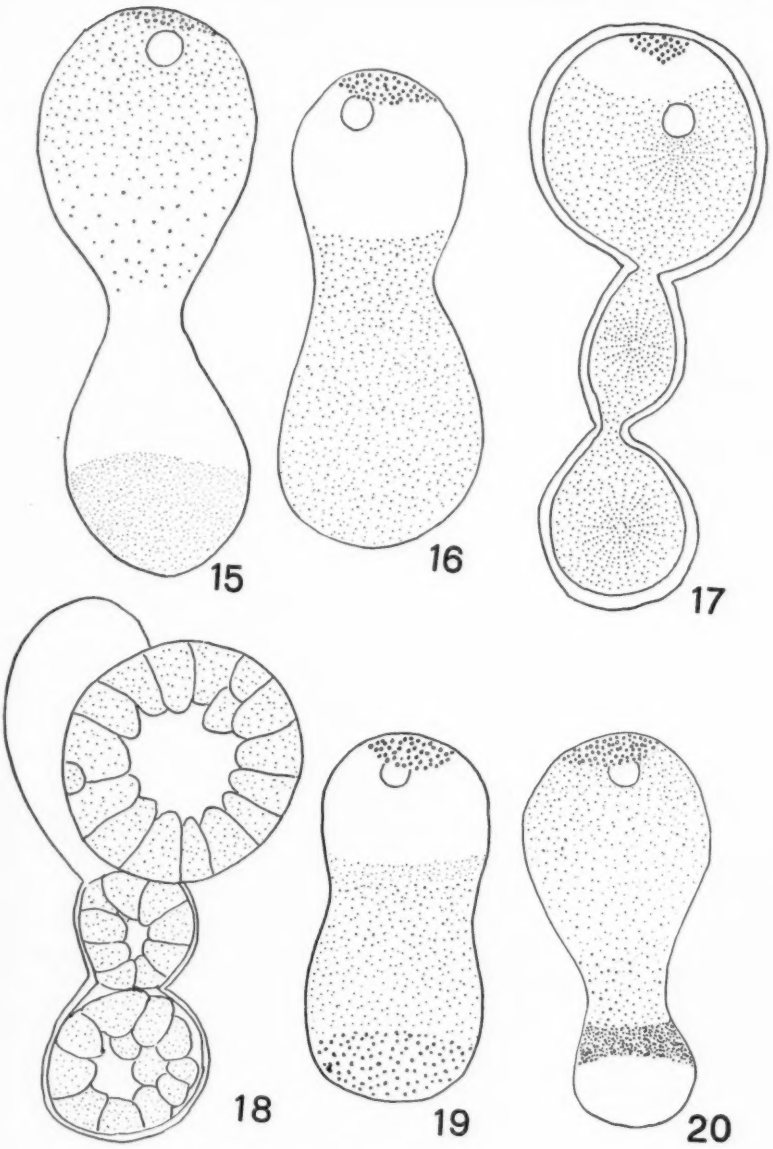
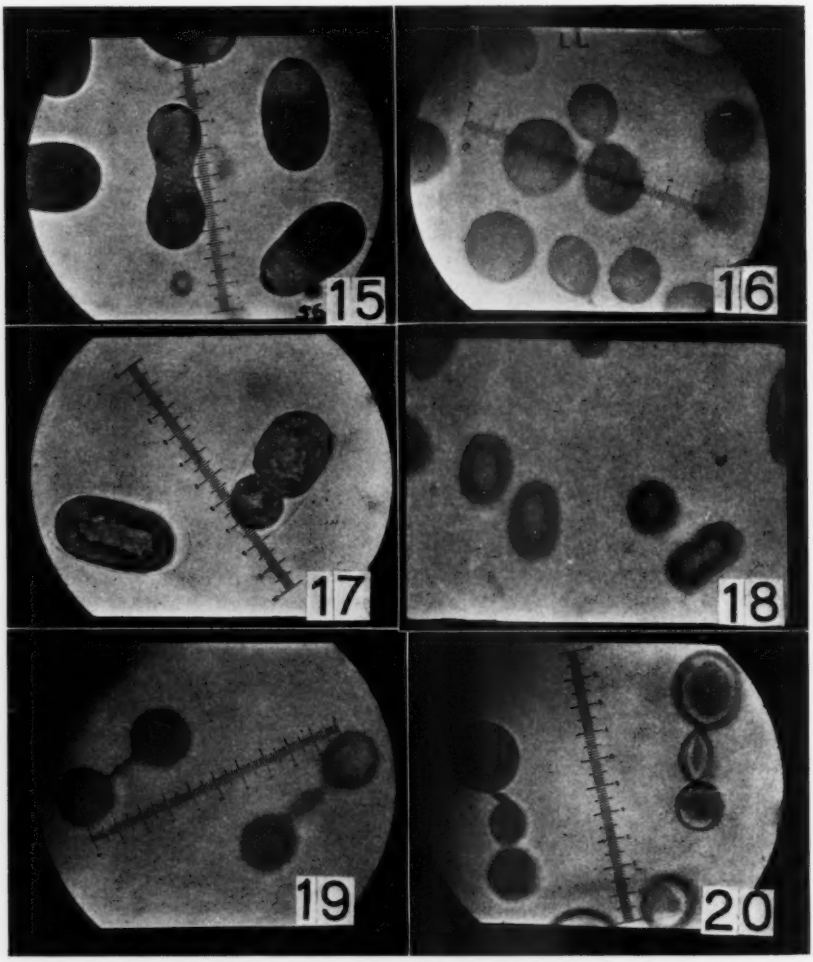


Plate III



Photographs 15–20. *Paracentrotus* (*Strongylocentrotus*) *lividus*

15. Whole egg.
16. Two half eggs in center; high speed.
17. Blastulæ of whole eggs, one at right from incompletely separated egg.
18. Blastula of granular half egg above at right, whole egg below it.
19. Two half eggs connected by strand; low speed.
20. Tripartite eggs. Egg has been separated into three parts instead of two.

force, and there is no massing of pigment at the heavy pole such as occurs in *Arbacia*. The band remains in place and is merely stretched as the egg elongates. It may be, therefore, in any relation to the stratification: parallel, diagonal, or perpendicular to it. In immature eggs with germinal vesicle, the red pigment goes to the light pole either with the oil or a little below it.

Half Eggs

The eggs break apart at about 9,000 r.p.m. into a smaller nucleated sphere consisting of oil, clear layer, and coarse granules, and a larger non-nucleate sphere consisting of a few coarse granules and fine granules (mitochondria?) (Photograph 16). The two spheres are often connected by a narrow strand (Photograph 19).

Speed and Size

The size of the two spheres varies with the centrifugal speed. The higher speed increases the size of the granular sphere as in *Sphærechinus* (Photograph 16). With slow speed the granular sphere is smaller and more nearly equal in size to the clear sphere (Photograph 19). The sizes with different speeds are given in Table II.

Development

Both the half eggs can be fertilized, throw off fertilization membranes well separated from the surface, and form swimming blastulæ (Photograph 18) and gastrulæ with skeleton and pigment. When the halves are connected by a strand, one or both parts may develop. The elongate whole egg tends to slip away from the fertilization membrane just after fertilization as in *Sphærechinus* and *Parechinus*. It develops into a slipper-shaped blastula, or, if constricted by centrifuging, into a more or less double blastula (Photographs 17, 18). Invagination always takes place in the reddish zone no matter where located. The gastrulæ are therefore of various shapes, elongate in the axis of the gut or flattened in this axis. There seems no doubt that the axis of the embryo depends upon the location of the red pigment band and not upon the stratification of materials or the elongation of the egg produced by centrifugal force. Owing to the difficulty in seeing the red pigment band in the half eggs, it was not possible to determine whether some of these entirely lacked the pigment band and therefore failed to gastrulate.

Triple Eggs

In one set of eggs centrifuged at 9,000 r.p.m. the egg constricted not into two spheres but into three of very constant and definite size: a

large sphere, a medium-sized sphere, and a small sphere between the two (Photograph 20). When fertilized, the membrane formed around all three parts and in each a sperm aster developed, indicating that each part was fertilized by a separate sperm (Fig. 17). Some of these developed into triplet blastulæ, the three parts developing independently and swimming together (Fig. 18). In one case, the two larger parts formed gastrulæ.

Arbacia pustulosa

Normal Egg

The egg of *Arbacia pustulosa* is very heavily pigmented, even more reddish than that of *Arbacia punctulata*. The diameter of the egg is 77–81 μ, averaging 79 μ; the nucleus measures 10 μ. The fertilization membrane is closely adherent to the egg, the perivitelline space measuring only 1–2 μ. The ectoplasmic layer is quite thick (3 μ on fertilization) and it is possible to tell by measuring the same egg before and after fertilization that it is formed on fertilization outside of and in addition to the red sphere. First cleavage takes place in 1 hour 50 minutes at 16°. The egg is quite dense; with jelly its density is about 1.101 (eggs evenly distributed in 1.1 m sucrose 4 parts : sea water 1 part), without jelly about 1.096 (eggs evenly distributed in 1.1 m sucrose 3 parts : sea water 1 part).[2]

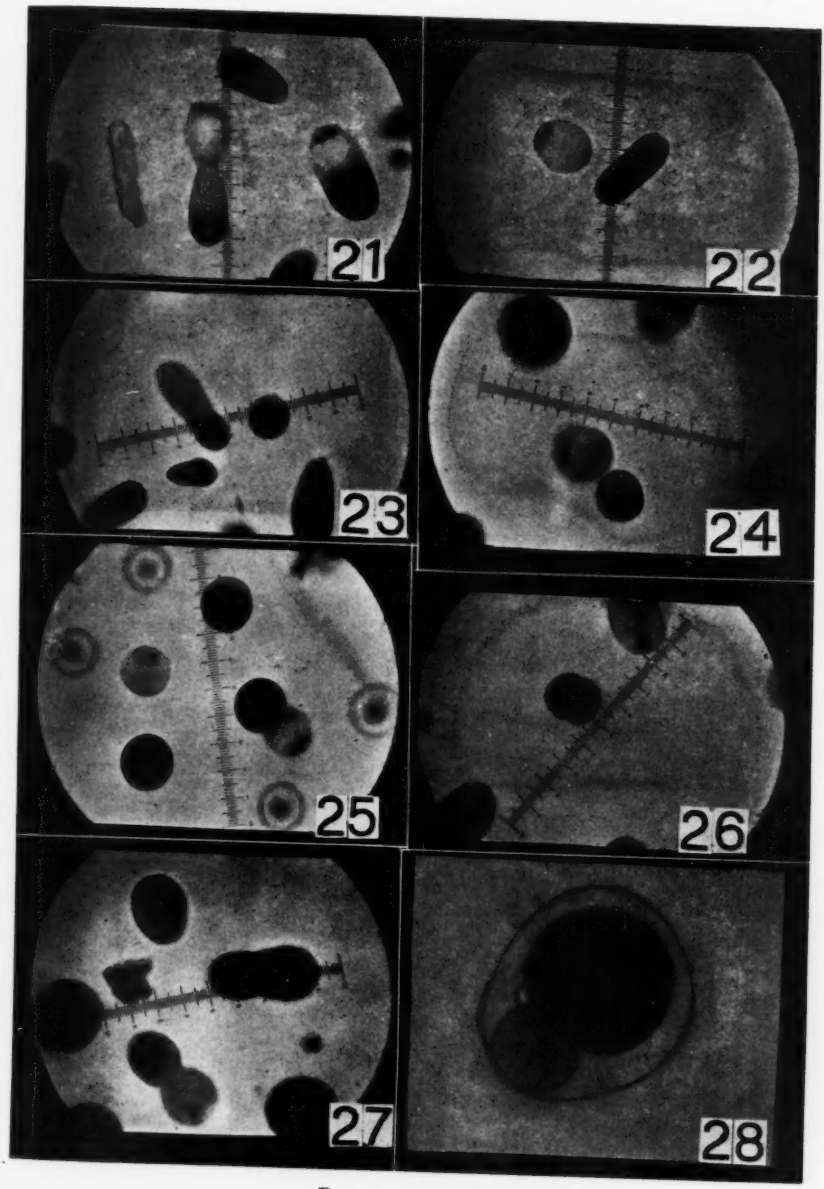
Centrifuged Egg

This egg breaks apart very readily and forms three layers in the centrifuge tube, distinct and well separated—the white nucleated spheres on top, the red non-nucleate spheres at the bottom, and dumb-bell-shaped whole eggs in the middle. The stratification is just as it is in *Arbacia punctulata* (1) oil, (2) clear layer in which lies the nucleus (3) fine granules or fifth layer (4) yolk granules and (5) red pigment (Fig. 19, Photograph 21). The granules measure approximately:

Oil	0.8 μ
Granules of fifth layer	0.5 μ
Yolk granules (polyhedral)	0.6–1 μ
Pigment	1.5 μ

Photographs 21–28. *Arbacia pustulosa*

21. Whole egg in center. Pigmented half above.
22. Two half eggs.
23. Pigmented half egg and the two quarters into which it breaks.
24. Low speed. White half larger than pigmented half.
25. High speed. Pigmented half larger than white.
26. Two-cell stage of pigmented half.
27. Blastula of white half above, of dumb-bell-shaped whole egg at right, and also below.
28. *Tripneustes* (*Hipponoë*) *esculenta*. Two half eggs within jelly.



PHOTOGRAPHS 21–28

The granules of the fifth layer stain purple with methyl green (mitochondria?).

Half and Quarter Eggs

The eggs are usually broken in 3 minutes at 9,000 r.p.m. across the yolk into a clear sphere containing oil, nucleus, clear layer and fifth layer (mitochondria?) and a little yolk; and a yolk portion containing yolk and pigment (Photographs 21–25). In some batches of eggs, the white sphere is a little larger than the red when centrifuged at 9,000 r.p.m. and in other batches the red sphere is a little larger than the white. The diameters of two typical lots centrifuged at 9,000 r.p.m. were: (1) whole egg 77 μ, nucleate half 62 μ, non-nucleate 60 μ; and (2) whole egg 78 μ, nucleate half 61 μ, non-nucleate 64 μ.

In one batch of eggs, broken apart at about 8,000 r.p.m., both the half eggs broke into quarters (Photograph 23, yolk quarters). The sizes of all halves and quarters are given in Table I.

Speed and Size

When the eggs are centrifuged at low speed, the white sphere is somewhat larger than the red sphere (Photograph 24); as the speed increases, the size of the red sphere increases, until at very high speed (about 13,000 r.p.m.) it is considerably larger than the white sphere (Photograph 25). At high speeds the egg breaks apart, giving two spherical half eggs (Photograph 25); the materials are not entirely segregated and the fifth layer is not very well formed. At low speeds, a much more definite segregation of materials takes place, the fifth layer is well formed, the two parts, especially the yolk half, become elongate before breaking apart (Photograph 21), and a tail is often left on the white sphere. The sizes of the two half eggs at different speeds in one lot of eggs are given in Table II.

Development

The deformed whole eggs and the half eggs of *Arbacia pustulosa* develop exactly as described for *Arbacia punctulata* (E. B. Harvey, 1932). The white nucleate halves divide quite regularly and give quite normal plutei with skeleton and arms but dwarf and lacking pigment. The red enucleate halves often develop without division planes coming in or represented by notches, but sometimes normal cleavage takes place (Photograph 26); and blastulæ and a few plutei with skeletons have been raised. These are not so normal or viable as the white halves. The whole eggs divide as in *Arbacia punctulata*, giving slipper-shaped blastulæ (Photograph 27) and later normal plutei.

TRIPNEUSTES (HIPPONOË) ESCULENTA

Normal Egg

The egg of *Tripneustes* (*Hipponoë*) *esculenta*, obtained in Bermuda in November, is slightly yellowish and very granular, similar in appearance to that of *Sphærechinus granularis*. It measures 82–86 μ, and is quite spherical when laid. The nucleus measures about 11.5 μ. The fertilization membrane adheres so closely to the surface that it is difficult to detect, leaving no perivitelline space. The ectoplasmic layer is very thin. The eggs develop slowly, taking 1½ hours for first cleavage after fertilization at 22° C.

Centrifuged Egg

The unfertilized egg breaks apart in about 15 minutes at about 8,000 r.p.m., 7 cm. radius. The stratification is usually (1) oil (2) coarse granules (3) fine yellow granules (4) clear layer (Fig. 20). The nucleus lies among the coarse granules under the oil cap. The granules measure approximately:

Oil	0.7 μ
Coarse granules	1.2 μ
Fine granules	0.7 μ

The fine yellow granules stain purple with methyl green (mitochondria?).

This egg, together with that of *Parechinus*, differs from the other sea urchin eggs, as well as most other eggs (except *Tubifex*), in having coarse granules under the oil, and the clear layer below. The coarse granules must, therefore, be lighter than the fluid medium of the egg. In *Parechinus*, the mitochondrial (?) granules are heavier than the medium, leaving the clear layer above these; in *Tripneustes* these granules, as well as the yolk granules, are lighter than the medium, leaving the clear layer below. By immersing the eggs in a medium of 70 per cent sea water: 30 per cent fresh water and centrifuging in this medium (with sucrose solution below), a very definite clear layer was formed under the oil. The medium in the egg, having been made less dense by the addition of water, is now lighter than the granules, and the granules sink to the heavier pole. In some batches of normal eggs and in some eggs of other batches, the clear layer is found, as in the eggs treated with dilute sea water, beneath the oil instead of at the heavy pole. When these eggs are centrifuged in concentrated sea water (1 gram NaCl + 100 cc. sea water), there is no clear layer beneath the oil. The medium of the egg having been made more dense, the granules now rise toward the lighter pole.

It may be noted in passing that eggs in dilute sea water break apart

much less readily than control eggs, and those in concentrated sea water much more readily.

Half Eggs

The *Tripneustes* egg breaks at about 8,000 r.p.m. (7 cm. radius) into two very unequal parts, usually a large sphere containing oil, nucleus, and coarse granules, and a very small sphere containing fine yellow granules (mitochondria?) and clear layer. The two parts are usually connected by a strand of tissue and are held together by the surrounding jelly, as shown by staining with Janus green (Photograph 28). The sizes of the parts are given in Table I.

Development

Both the nucleate and non-nucleate half eggs can be fertilized, the fertilization membranes closely adhering to the surface. The large sphere divides equally, sometimes through the oil cap, sometimes elsewhere. Swimming blastulæ develop a little later than those from whole eggs, then form gastrulæ which later obtain a skeleton and pigment. The small spheres develop with only the male nucleus, sometimes division planes come in, but usually only the nuclei divide until blastulæ are formed. These swim still later than those from the larger half egg. The two halves usually break their connecting strand when becoming free-swimming. The larger halves are much more viable and normal than the smaller halves.

Cross-fertilization

Some experiments were done with fertilizing the half and quarter eggs of one species with the sperm of another species. In general it was found that when cross fertilization is not possible (in sea water) with normal eggs, it is also not possible with centrifuged eggs nor with half and quarter eggs. When cross-fertilization is possible with normal eggs, it takes place in about the same percentage (sometimes slightly more) in the deformed whole eggs and in the half and quarter eggs, both nucleate and enucleate. The best results were with *Sphærechinus* ♀ and *Paracentrotus* ♂, where many quite normal cleavages occurred in all types of eggs. Some of these hybrids from fertilized enucleate half eggs were raised to plutei with skeletons and have lived as long as seven days.

Discussion

A comparison of the results obtained in five species of sea urchin brings out several facts. On the one hand, there are marked differ-

ences in the normal egg of the different species. They differ in size, density, size of nucleus, size of perivitelline space, thickness of ectoplasmic layer, and rate of development. On centrifuging, there is a difference in the distribution of visible materials (except in the two species of *Arbacia*), in the force necessary to break the eggs apart, in the size of the corresponding portions and in their viability after fertilization. On the other hand, there are certain similarities in the eggs. All the eggs stratify when centrifuged, become dumb-bell-shaped, and break into two spheres of very definite size. The granules which are moveable by centrifugal force, though differing in relative size and weight, are probably fundamentally the same in all the eggs: oil, yolk granules, and another kind of granules, probably mitochondria; in the two species of *Arbacia* there are pigment granules in addition.

When the eggs are broken by centrifugal force into halves or quarters, all the parts can be fertilized, and form fertilization membranes characteristic of the particular normal whole egg. All the parts are capable of development, some of the parts of complete development, at least as far as plutei. The female nucleus does not seem to be essential for development, since in some cases the half lacking this nucleus (androgenetic) develops better than the half containing it. I have found no evidence of any localization of organ-forming material around the nucleus, as found by Harnley (1926). The visible granules do not seem to be the essential materials for development, since the fundamental organs such as gut and skeleton can be laid down in the absence of any one special type of granule. These granules seem to be accessory rather than essential materials in development; the essential material for development seems to be the "ground substance." This agrees with the conclusions of many others who have worked on centrifuged eggs (especially Morgan) and with the work of E. B. Wilson (1929) on the centrifuged fragments of *Chætopterus* eggs. There seems to be little localization of visible organ-forming materials in the unfertilized sea urchin egg, a conclusion reached also by Plough (1929) and by Tennent, Taylor, and Whitaker (1929).

The pigment band characteristic of the *Paracentrotus* egg belongs to a different category from the other visible materials, as this is *not* displaced by centrifugal force and *does* indicate the place of invagination, whether or not it is actually gut-forming material. This agrees with the earlier work of Boveri (1901) and the later work of Hörstadius (1928) on this egg, definitely locating the gut-forming material in this band.

SUMMARY

1. The unfertilized eggs of *Sphærechinus granularis*, *Parechinus* (*Echinus*) *microtuberculatus*, *Paracentrotus* (*Strongylocentrotus*) *lividus* and *Arbacia pustulosa* from Naples and of *Tripneustes* (*Hipponoë*) *esculenta* from Bermuda have been stratified and broken into halves and some of these halves into quarters by strong centrifugal force.

2. The relative size of the halves and their granular content is fairly constant for a definite centrifugal speed, but the size varies with the speed.

3. The visible granules are stratified with centrifugal force; the stratification is differently arranged in the different species but always includes oil, yolk and mitochondria (?). The pigment band of the *Paracentrotus* egg is not displaced but only stretched.

4. When fertilized, all of the halves and quarters, both nucleate and non-nucleate, form fertilization membranes and develop, some into normal dwarf plutei.

5. When the half eggs are connected by a strand of tissue, either one or both spheres may be fertilized by a sperm and either one or both may develop.

6. In one batch of *Paracentrotus* eggs, the eggs constricted into three parts instead of two, and each part received a sperm and developed.

7. Half and quarter eggs, both nucleate and non-nucleate, can be cross-fertilized in approximately the same percentage as the whole eggs. Some of the cross-fertilized non-nucleate halves were raised to plutei.

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STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

I. Chemistry of the Waters of the Gulf of Maine in August, 1932

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The hydrographic features of the Gulf of Maine—temperature, salinity, and the dominant concentration of its waters—are now known broadly speaking (4) (5). The same may be said of the general occurrence of its principal inhabitants (3). The final goal of such studies, the explanation of the natural economy of these inhabitants in terms of their physical and chemical environment, lies ahead. It is to be sought not so much by the generalized surveys of the area as by more detailed study of the physical and, more especially, of the chemical changes in the water from season to season at selected locations, combined with simultaneous observations on the occurrence, abundance, and distribution of the animals and plants.

Oceanic biologists in various parts of the world have devoted much attention during the past few years to the factors and events that control the biologic fertility of the seas. Parallelism has so repeatedly been established between variations in the abundance of phytoplankton on the one hand and in the available supply of plant-nutrients on the other, that a causative relationship is now universally accepted. Much of detail is yet to be learned not only regarding the nutritive requirements of different groups of planktonic plants, but equally as to the situation existing with regard to the cycle of limiting substances in different parts of the sea.

The Gulf of Maine appears to be a particularly favorable area for such detailed studies. Within a circumscribed region of ready accessibility there occurs a variety of hydrographic conditions, in many ways representative of boreal seas as a whole. These range from the turbulent shallow waters overlying the offshore banks to the stable and stratified waters which develop in the deeper portions of the Gulf during the summer. Within this area exceptionally large ranges of temperature

[1] Contribution No. 8, Woods Hole Oceanographic Institution.

occur, both seasonal and in correlation with the depth of the water. Furthermore, the deeper portions of the Gulf appear to be fed by a tongue of water originating at even greater depths offshore. This water will be shown from its chemical content to be characteristic of the more stagnant waters of the deep sea. Conditions are consequently favorable for the examination of the nutrient cycle in all its phases.

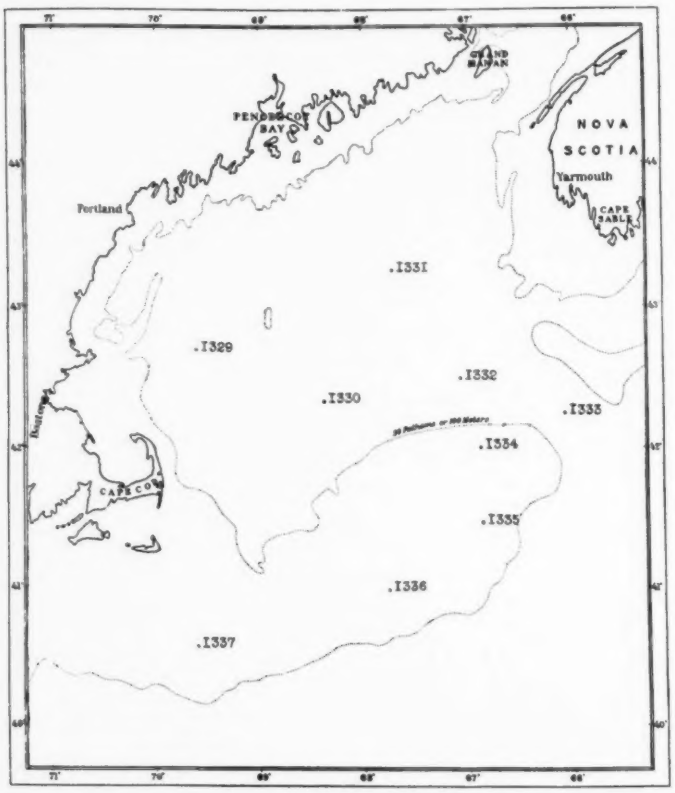

FIG. 1. Locations of "Atlantis" Stations 1329–1337, August 2–5, 1932.

During the month of August, 1932, a cruise of the research ship "Atlantis" afforded an opportunity for the simultaneous study of the quantitative distribution of phytoplankton, of bacteria, and of the most obviously important nutrient substances of the sea water. Stations were made in two regions of striking contrast, the deep basin of the Gulf on the one hand, and the waters over its shoal southern rim (Georges Bank)

on the other. The location of the stations is charted in Fig. 1 and their position recorded in Table I.

The present paper deals with the chemical observations made during this cruise. The results of the study of phytoplankton and bacteria are presented in the succeeding communications.

The chemical program of the cruise consisted in the determination of phosphate, nitrate, nitrite, and oxygen in the water at various depths from all stations. The methods used were those which have now become rather well standardized: for phosphate, the colorimetric molybdic acid method successfully used by Atkins and many others (1) (2) (3) (8); for nitrate, the colorimetric method of Harvey (7) (8), using reduced strychnine; for nitrite, the Griess-Ilosvay method, but substituting the diethyl derivative of alpha-naphthylamine; for oxygen, Winkler's iodimetric titration. All analyses were carried out on ship-board, as

TABLE I

Location of Stations

Station No.	Date	Lat. N.	Long. W.
1329	August 2, 1932	42° 42′	69° 36′
1330	August 2, 1932	42° 19′	68° 22′
1331	August 3, 1932	43° 14′	67° 45′
1332	August 3, 1932	42° 28′	67° 03′
1333	August 4, 1932	42° 15′	66° 04′
1334	August 4, 1932	42° 00′	66° 54′
1335	August 4, 1932	41° 26′	66° 53′
1336	August 5, 1932	40° 57′	67° 49′
1337	August 5, 1932	40° 35′	68° 42′

soon as possible after the water samples had been taken. The oxygen and phosphate analyses were made by Mr. T. S. Greenwood.

From the chemical standpoint as well as the hydrographic the stations may be divided into two groups; the first five, Nos. 1329–1333, fairly deep, in the Gulf of Maine; and the last four, Nos. 1334–1337, much shallower, on Georges Bank. A more thorough mixing is characteristic of the latter group, a less pronounced thermocline and, as a consequence, a more nearly homogeneous water mass, top to bottom.

The data are given in Table II and plotted in depth-concentration curves, Figs. 2, 3, and 4. In the latter, the scale is different for each of the five items, and is so chosen as to make the curves most nearly correspond with each other. In the tables and curves the lower limit for nitrite nitrogen is taken as approximately 0.4 mg./m.^{3}; that is, the water is considered nitrite-free when amounts less than this are present, although with care it is possible to detect perhaps half this amount.

TABLE II

Hydrographic and Chemical Data for Stations in the Gulf of Maine and on Georges Bank

	Depth	Temp.	Salinity	Nitrate N	Nitrite N	Phosphate PO_4	Oxygen content	Oxygen saturation
	meters	*° C.*		*mg./m.³*	*mg./m.³*	*mg./m.³*	*cc.*	*per cent*
	1	19.19	32.00	25	0	18	5.40	98
	10	16.81	32.21	24	0	13	6.30	110
	20	11.02	—	35	0	11	7.27	113
	30	8.16	32.45	34	0	49	7.44	108
Station 1329	40	6.2	32.63	120	1.3	77	6.63	94
August 2, 1932	50	5.15	32.92	175	1.0	102	6.39	89
	60	5.2	32.79	195	1.0	100	6.34	88
	75	4.83	32.92	240	1.0	115	6.10	84
	100	4.59	33.19	275	0.8	85	5.79	80
	240	6.30	34.23	425	1.1	96	4.38	63
	1	19.81	32.70	21	0	20	5.56	102
	10	19.43	32.20	21	0	11	5.57	102
	19	13.11	32.54	25	0	11	6.99	114
	29	8.80	32.66	27	0	36	7.26	109
Station 1330	39	6.03	32.92	105	1.6	—	6.75	96
August 2, 1932	48	5.01	32.92	170	0.4	43	6.51	90
	63	4.65	32.92	190	0.4	45	6.46	89
	77	4.45	33.01	240	0.7	54	6.34	87
	101	4.47	33.12	250	0	59	6.17	85
	189	5.81	34.23	390	0	82	4.76	68
	1	16.57	32.75	28	1.2	12	6.07	106
	10	13.87	32.39	27	1.3	8	6.63	110
	19	9.65	33.03	35	2.1	9	7.24	111
	29	8.54	32.77	75	2.9	18	6.53	97
Station 1331	39	6.79	32.92	135	3.6	17	6.07	87
August 3, 1932	48	5.81	32.92	150	1.7	18	5.94	83
	58	6.00	33.12	210	1.4	19	5.78	82
	72	5.68	33.13	225	1.2	80	5.67	80
	97	5.53	32.97	285	0.9	—	5.43	76
	194	6.67	34.37	350	1.6	—	4.47	65
	1	17.67	32.48	22	0	22	5.90	104
	9	17.60	32.72	20	0	22	5.90	104
	19	13.90	32.34	16	0	23	6.66	110
	28	11.09	32.66	27	1.5	36	6.95	107
	38	9.67	32.57	27	1.4	39	6.81	104
Station 1332	47	8.65	32.59	60	2.7	50	6.82	102
August 3, 1932	56	7.31	32.84	155	3.8	67	6.40	93
	71	6.13	33.68	235	1.5	86	5.94	84
	94	6.13	33.89	265	1.2	100	5.39	77
	188	7.17	34.85	450	1.8	132	4.27	63
	310	6.33	34.85	500	1.1	138	4.48	64

TABLE II (*Continued*)

	Depth	Temp.	Salinity	Nitrate N	Nitrite N	Phosphate PO_4	Oxygen content	Oxygen saturation
	meters	*° C.*		*mg./m.³*	*mg./m.³*	*mg./m.³*	*cc.*	*per cent*
	1	17.31	32.43	16	0	22	5.78	102
	9	16.81	32.34	20	0	28	6.00	104
	17	9.96	32.48	20	0	32	6.92	106
	26	7.57	——	80	3.5	36	6.68	98
Station 1333	35	6.76	32.70	110	2.5	54	6.60	94
August 4, 1932	44	6.10	32.83	150	1.7	73	6.40	90
	52	6.00	33.44	175	0.9	70	6.39	91
	65	5.61	33.44	240	3.7	81	5.94	84
	87	6.00	34.02	275	2.7	97	5.39	77
	186	6.67	34.90	460	3.7	100	4.69	68
	1	14.98	32.52	20	0	25	6.10	103
	9	15.02	32.48	27	0	34	6.11	103
	17	14.15	32.66	24	0	38	6.12	102
Station 1334	26	13.25	32.70	40	1.8	43	6.17	101
August 4, 1932	35	12.55	32.70	45	1.9	35	6.24	101
	43	11.86	32.74	60	2.0	37	6.22	99
	52	9.01	32.79	100	2.5	38	6.28	95
	1	15.50	32.54	25	1.9	26	6.34	108
	10	15.45	32.38	25	2.6	—	6.35	108
	20	12.90	32.74	25	1.4	—	6.68	109
Station 1335	30	10.61	32.88	65	4.0	71	6.24	97
August 4, 1932	40	10.04	32.79	80	4.1	64	6.18	95
	50	10.26	33.01	80	3.9	67	6.18	96
	60	10.11	32.74	85	4.0	62	6.17	95
	1	15.23	32.70	21	1.0	31	6.25	106
	10	14.35	32.66	22	1.2	32	6.31	106
	20	11.07	32.70	60	3.2	35	6.22	98
Station 1336	30	10.70	32.66	70	3.9	45	6.12	95
August 5, 1932	40	10.65	32.68	70	3.5	37	6.06	94
	50	10.63	32.66	70	3.4	—	6.17	96
	60	10.63	32.74	75	3.9	63	6.10	95
	1	19.85	32.95	26	1.6	29	5.82	107
	10	17.97	32.63	24	1.1	31	6.12	109
	20	11.49	32.68	24	0.6	28	7.24	114
Station 1337	30	9.13	32.81	95	7.4	76	5.96	90
August 5, 1932	40	8.98	33.44	105	7.4	69	5.95	90
	50	8.97	32.83	105	6.9	77	5.95	90
	60	8.97	32.83	105	7.0	82	5.89	89

Oxygen.—The figures for dissolved oxygen content agree in general with those found in other similar investigations. When the " percentage of saturation " is calculated it is found that the upper 20–40 meters are invariably " supersaturated," with a maximum corresponding to the level

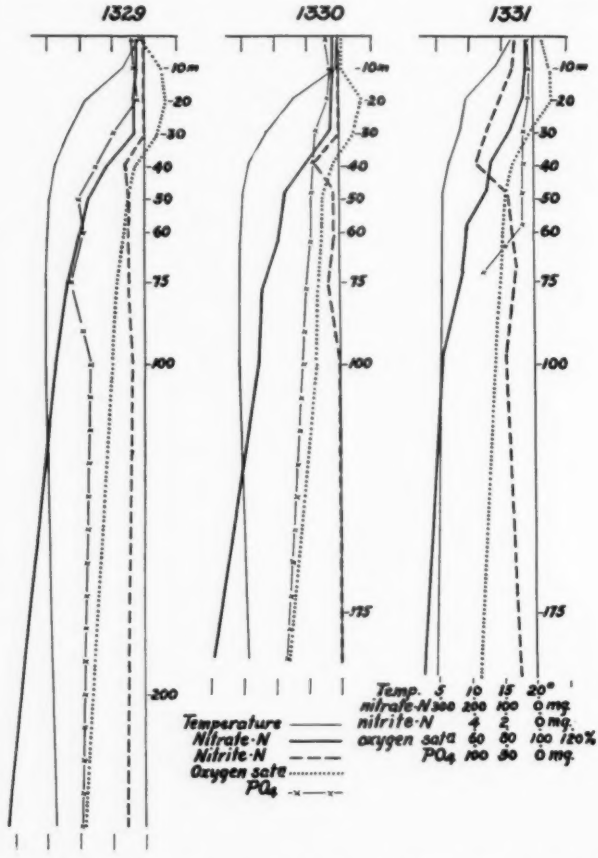

FIG. 2. Chemical data for stations in the deep basin of the Gulf of Maine, August, 1932.

of greatest diatom abundance. This is brought out in detail in the section of this report dealing with diatom distribution. In no case is there any clearly defined minimum at an intermediate depth, but always a continuously decreasing percentage to the bottom.

Phosphate.—The phosphate values were obtained under rather less than ideal conditions but are satisfactory for comparative purposes. Only at the first three stations do the concentrations approach critically low values, and it is to be observed that the minimum quantities are

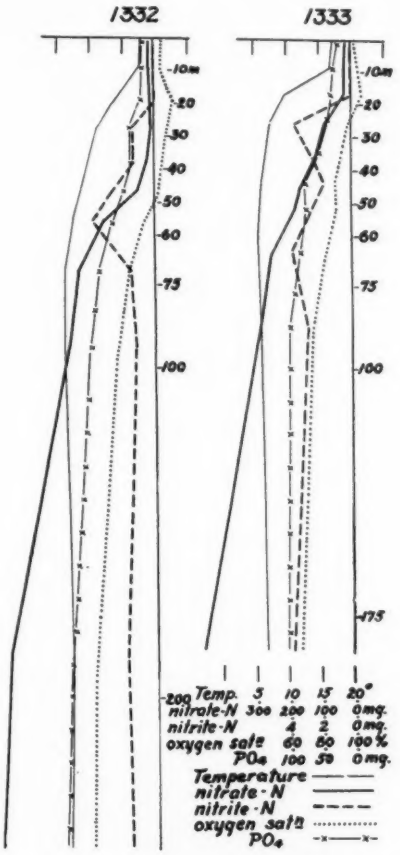

FIG. 3. Chemical data for stations near the Eastern Channel, August, 1932.

found at depths of 10–20 meters, corresponding to the regions of highest oxygen saturation.

Nitrate.—The concentrations of nitrate nitrogen do not in any instance approach what have been considered minimal quantities. However, the correlation of oxygen saturation and diatom abundance with

nitrate is much closer than with phosphate. The depth-concentration curves indicate how almost every change in oxygen is accompanied by a similar one in nitrate. The concentrations of nitrate reported here will be observed to be somewhat higher than those generally recorded in the literature. But four years of experience with the method leads the author to believe them fairly reliable. Each sample was determined in duplicate, and the color developed by the reagent compared with a set of dye-standards twice standardized against a series of sea-water samples with known amounts of added nitrate. Deep water samples were diluted with surface water of known nitrate concentration to bring them within the most reliable range of the standards. Anyone who has worked with the method will realize the uncertainties involved in determining absolute amounts of nitrate, although relative values are doubtless satisfactory.

Nitrite.—The nitrite determinations made on this cruise are but one part of a larger study of the variations and general significance of nitrite

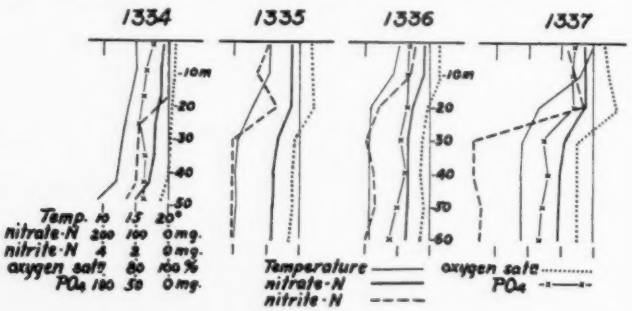

FIG. 4. Chemical data for stations on Georges Bank, August, 1932.

in the sea, which will be reported later. The position which nitrite occupies in the general nitrogen cycle in the sea has been pointed out on several occasions (9) (10) (7) (11 and others). Its occurrence in sea water, under many circumstances at least, is altogether certain, notwithstanding Harvey's doubts, expressed in 1926 (7). Since it occupies an intermediate position in the oxidation of ammonia to nitrate, its concentration might naturally be expected to be exceedingly variable, depending upon conditions. In fact, there are several agencies, both chemical and biological, which can alter it. The results of Atkins (11) and several others, including the present author, show conclusively that the presence of nitrite is not in any high degree dependent upon prox-

imity to the shore, even in cases in which pollution may be expected. Atkins' assumption (11) as to the significance of the nitrite concentration (namely, that it is an index of the extent of the nitrification process) is hardly adequate. That this is too simple an explanation of its variations may be seen even from the few data here reported, and more particularly on consideration of the bacteriological results in a later section of this report.

Under what may be considered normal conditions at this time of year, the sea water is devoid of nitrite in the upper levels. In only one of the five deep stations, No. 1331, do we find an exception to this, and this station, somewhat isolated in the northern basin of the Gulf, differs from the other stations in further respects. Its thermocline is not so pronounced, and the low phosphate values extend further down. When nitrite is found at the surface it is usually associated with rather large amounts lower down and with evidences of vertical mixing. This can be seen in the depth-concentration curves for Stations 1334–1337.

Another feature of the present investigation, which has been observed by the author on a number of other occasions, is the occurrence of a maximum nitrite concentration somewhere in the neighborhood of 40 meters. The finding of such intermediate maximum values and Waksman's establishment of the fact that the majority, if not all, of the oxidation of ammonia takes place in the bottom, show the inadequacy of regarding nitrite as a mere index of nitrification. Furthermore, Waksman's discovery of nitrate-reducing organisms in the upper levels, and their relation to the diatoms, make it very likely, as will be shown in the third paper of this series, that the nitrite maximum is due to reduction of nitrate rather than to oxidation of ammonia.

The actual bottom water differs in nitrate and nitrite content from that obtained in the lowest sample bottle. The water brought up with sand in the Peterson Grab at Station 1336 was filtered off and found to have a concentration of nitrate nitrogen of 350 mg./m.^{3}, and of nitrite, 25 mg. This may be compared with the nitrate, 75 mg., and nitrite, 3.9 mg., found in the sample at 60 meters, only four meters above. Approximately the same ratio between nitrate and nitrite exists in the two cases, but the enormous difference in the absolute quantities shows clearly where the active nitrification is going on. Again, mud obtained from Station 1331, when washed with nitrite-free surface water, yielded nitrite to the extent of 8.8 mg./m.^{3}, as compared with 1.6 mg. in the lowest water sample. Other similar cases from different areas will be found in a subsequent paper on this general problem.

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STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

II. Distribution of Phytoplankton in August, 1932

H. H. GRAN [1]

The general distribution of the species of phytoplankton in the Gulf of Maine is well known from Bigelow's papers (1915, 1928). The summer conditions are described (1928, p. 391) as follows:

"In midsummer we have usually found the entire basin of the gulf occupied by a peridinian (Ceratium) plankton, with only occasional diatoms, and we have never found diatoms in abundance anywhere in the gulf in July or August except close along the coast, on the one hand, and on Georges Bank, on the other.

"We found diatoms flowering in abundance on each of our summer visits to the latter locality, but in different regions in different years. Thus they dominated on the western end of the bank on July 9, 1913, and on July 23, 1916, but when we visited that general locality on July 20, 1914, the water contained very few diatoms but, instead, a characteristic peridinian plankton. Three days later, however, we encountered a rich flowering of diatoms near the northeastern edge of the bank. Furthermore, the Georges Bank flowerings of July, 1913 and 1914, though far apart geographically, were both dominated by Guinardia. In July, 1916, however, we found no Guinardia on a traverse of the western part of the bank but swarms of Thalassiothrix and Rhizosolenia in its stead."

. . . "It is not yet clear whether any particular region on the banks is more favorable for the multiplication of diatoms than another, except that we have always found these rich flowerings on its shoaler parts and never close enough to the continental slope to be within the influence of the high temperature outside the edge, which in its own turn, supports various oceanic diatoms in small numbers mingled with peridinians of similarly Tropic origin."

During the cruise with the "Atlantis" in the beginning of August, 1932, we visited the same localities and found on the whole the same general distribution of the communities as in the previous years. The newer methods allowed us to study quantitatively the horizontal and vertical distribution of the various species in correlation with chemical and physical factors, and thus to supplement the previous observations.

[1] Contribution No. 9, Woods Hole Oceanographic Institution.

At each station water samples for centrifuging were collected from the same water bottles giving samples for chemical determinations. To each sample of 150 cc. were added 5 cc. of a neutralized formalin solution (equal parts of *pure* 40 per cent formalin and distilled water, neutralized by adding drops of a strong sodium carbonate solution to neutral reaction controlled by litmus paper, and carefully filtered before use). Of each sample 55 cc. were centrifuged, and calculated as 50 cc., because experience has showed that about 10 per cent of the content is lost as adhering to the glasses of the centrifuge and to the pipette, even if these are carefully cleaned by rinsing with chromic acid between each operation. The sediments of the four tubes of the centrifuge were carefully transferred to one tube with acute bottom and centrifuged once more. The clear water above the sediment was sucked off cautiously by a pipette and only a sufficient quantity left to be covered by a cover-glass 20 mm. square. The slide is ruled across with 0.4 mm. distance between the lines. For the counting a 4 mm. apochromate (Zeiss) was used. With a compensation ocular No. 4 two intervals are covered by the field of the microscope, with ocular 8 just one (0.4 mm.). The whole content below the cover-glass must be counted, as it is impossible to get the sediments evenly distributed. Every cell is counted also of species forming chains. When one or two chain-forming species are very abundant, it may be sufficient to count the number of chains and calculate the number of cells by multiplication with the average number of cells per chain, separately determined in that same sample. In the tables the frequency of each species is given as cells per liter, the figure of 20 thus representing one cell observed in 50 cc. of sea water.

Vertical hawls with No. 20 silk nets were taken at all stations and used for a preliminary investigation. As a rule all species observed in the net samples were also found in the centrifuged water samples except larger forms as e.g. *Acanthometron*, which was abundant at some of the stations in the deeper part of the Gulf, as also found by Bigelow in 1914. Further a number of nannoplankton species were observed which could not be caught by the nets.

Of the eight stations from which phytoplankton samples were examined, the first five (1329–1333) were taken in the deeper part of the Gulf of Maine, the last three (1334–1336) on Georges Bank. The two groups are best described separately, as they show considerable differences in the quantitative distribution of the species.

The positions of the stations and the data concerning the chemical condition of the sea water are given in the preceding paper (Rakestraw, 1933).

The Deep Basin of the Gulf of Maine

All stations in this area show a marked stratification with regard to the hydrographical conditions as well as to the vertical distribution of the plankton. The surface is covered, downwards to 10 meters or more, by

Table I

"Atlantis" Station 1330, August 2, 1932

Depth, *meters*	1	10	19	29	39	48	77
Temperature, ° *C*	19.81	19.43	13.11	8.80	6.03	5.01	4.45
Oxygen, *cc. per liter*	5.56	5.57	6.99	7.26	6.75	6.51	6.34
Oxygen, *% of saturation*	102	102	114	109	96	90	87
Nitrate, *mg. N per cu. m.*	21	21	25	27	105	170	240
	cells per liter	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
Halosphæra viridis juv	—	—	340	—	—	—	—
Coccolithus pelagicus	220	140	—	—	—	—	—
Pontosphæra Huxleyi	260	220	6640	37200	300	—	—
Dictyocha fibula	—	—	20	80	—	—	—
Actinocyclus Ehrenbergi	—	—	20	—	180	160	120
Corethron hystrix	—	—	—	40	—	20	—
Coscinosira Œstrupi	—	140	—	220	720	160	60
Nitzschia seriata	—	—	40	600	280	—	—
Rhizosolenia alata	40	120	180	160	—	60	—
Rhizosolenia Shrubsolei	—	—	—	40	160	—	—
Ceratium bucephalum	—	—	—	—	140	460	60
Ceratium fusus	20	40	240	360	20	—	—
Ceratium longipes	20	—	20	20	60	280	80
Ceratium tripos	20	—	—	—	—	—	—
Exuviælla baltica	40	100	120	100	—	—	—
Gonyaulax orientalis	20	—	—	40	—	20	—
Gonyaulax orientalis, cystæ	20	40	240	660	40	80	20
Acanthostomella norvegica	—	—	—	20	20	—	—
Labœa conica	20	20	—	—	—	—	—
Labœa strobila	40	60	—	—	—	—	—

The following species were found in small numbers:

Cerataulina Bergonii 30 m., 20. *Chætoceros decipiens* 50 m., 60. *Coscinodiscus centralis* 30 m., 20. *C. excentricus* 30 m., 40. *Leptocylindrus danicus* 30 m., 40. *Nitzschia delicatissima* 30 m., 100. *Thalassiosira decipiens* 40 m., 40. *T. gravida* 30 m., 40.

Oxytoxum gracile 20 m., 20. *Peridinium brevipes* 10 m., 20. *P. Steinii* 10 m., 20. *Protoceratium reticulatum* 30 m., 20.

Favella denticulata 75 m., 20. *Lohmanniella spiralis* 10 m., 20. *Ptychocylis obtusa* 30 m., 20.

a layer of high temperature, mostly above 15°, and low specific gravity (σ_t less than 25). These surface layers were less saturated with oxygen than the transition layers in the depths of 20–30 meters, which indicates that the photosynthesis of the phytoplankton had been more active at 20

TABLE II

"Atlantis" Station 1331, August 3, 1932

Depth, *meters*	1	10	19	29	39	48	72
Temperature, ° *C*	16.57	13.87	9.65	8.54	6.79	5.81	5.68
σ_t	23.91	24.22	25.49	25.47	25.83	25.96	26.13
Oxygen, *% of saturation*	106	110	111	97	87	83	80
Nitrate, *mg. N per cu. m.*	28	27	35	75	135	150	225
	cells per liter	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
Coccolithus pelagicus	—	—	—	—	20	20	20
Pontosphæra Huxleyi	2060	940	80	—	—	—	—
Distephanus speculum	—	—	—	120	—	40	—
Actinocyclus Ehrenbergi	—	—	—	40	20	20	—
Cerataulina Bergonii	—	—	160	280	—	—	—
Chætoceros borealis	—	—	—	—	160	20	—
Chætoceros compressus	—	—	—	1680	1060*	20*	40*
Chætoceros constrictus	—	—	320	3460	2700*	60	—
Chætoceros decipiens	—	—	—	360	500	40	140
Chætoceros diadema	—	—	—	700	200	—	—
Chætoceros laciniosus	—	—	—	440	160	—	—
Chætoceros radicans	—	—	—	—	80*	—	40*
Corethron hystrix	—	—	—	180	20	—	—
Coscinodiscus excentricus	—	—	—	—	40	20	—
Coscinosira Œstrupi	—	480	60	1800	1520	380	—
Leptocylindrus danicus	—	120	—	120	360	—	—
Rhizosolenia alata	1480	1780	700	200	220	20	20
Rhizosolenia Shrubsolei	—	—	100	200	120	—	—
Thalassiosira gravida	—	—	20	40	20	—	—
Ceratium bucephalum	—	20	260	440	40	—	—
Ceratium fusus	—	20	500	280	—	—	—
Ceratium lineatum	240	—	—	—	—	—	—
Ceratium longipes	—	20	—	80	380	200	40
Ceratium tripos	20	20	280	20	—	—	—
Dinophysis norvegica	20	—	20	—	—	—	—
Exuviælla baltica	—	—	—	—	40	60	120
Gonyaulax orientalis	20	—	280	80	—	—	—
Gonyaulax orientalis, cystæ	—	180	1060	740	100	20	20
Peridinium brevipes	—	—	20	20	20	—	—
Peridinium ovatum	—	—	—	20	80	100	—
Peridinium pellucidum	—	—	—	60	—	20	—
Peridinium Steinii	—	20	120	180	20	—	—
Favella denticulata	—	—	20	20	20	—	—
Lohmanniella oviformis	—	—	20	20	20	—	—
Ptychocylis obtusa	—	—	—	80	60	40	—
Tintinnopsis sp	—	—	—	80	80	—	—

The following species were observed in small numbers: *Chætoceros affinis* 30 m., 140; *Ch. convolutus* 30 m., 60. *Ch. debilis* 40 m., 360. *Ch. teres*, resting spores 40 m., 80; *Coscinodiscus radiatus* 40 m., 20. *Rhizosolenia fragilissima* 30 m., 160; *Rh. styliformis* 50 m., 20. *Skeletonema costatum* 30 m., 300. *Thalassiothrix longissima* 30 m., 60.

Gonyaulax spinifera 10 m., 20. *Peridinium depressum* 75 m., 40. *P. pyriforme* 30 m., 40. *Acanthostomella norvegica* 20 m., 20.

* with resting spores.

to 30 meters than at the surface. This also agrees with the fact that the phytoplankton had its maximum of frequency just in the same depths, 20 to 30 meters, as shown by Tables I and II, in which the results of the countings are represented for two selected stations, 1330 and 1331.

The dominating species were *Ceratium fusus*, *Pontosphæra Huxleyi* and germinating cystæ of *Gonyaulax orientalis*. The diatoms were represented by *Rhizosolenia alata*, which also elsewhere as a rule accompanies the Ceratium-plankton during the summer as a subordinate part of the phytoplankton, and by the little *Coscinosira Œstrupi*. Also *Nitzschia seriata* and *Rhizosolenia Shrubsolei* occurred in small numbers. Neritic diatoms were scarce, and some of them had resting spores as a sign that they may be regarded as the last remains of a population from an earlier season. At Station 1331 they were found in greater numbers than elsewhere, with a distinct maximum at 30 meters depth and an accumulation of resting spores at 40 meters. Just at this station

TABLE III

Ceratium bucephalum, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	—	—	—	—	—	—	—	20
10	—	—	20	—	—	—	—	—
20	20	—	260	—	20	—	—	—
30	20	40	440	—	—	—	20	—
40	60	140	40	—	—	40	—	—
50	100	460	—	—	—	40	—	—
75 (60)	—	60	—	—	—	—	—	—

the warm surface layer was shallower than at the other stations, the temperature was below 10° from 20 m. and downwards, and the high nitrate content characteristic of the deeper layers was found right up to 30 meters depth (75 mg. N per cu. m.), just where these diatoms had their maximum. Also at this station the surface layer itself was somewhat richer than elsewhere in the Gulf; *Pontosphæra*, *Rhizosolenia alata*, and *Ceratium lineatum* had their maxima above 10 meters depth.

At all stations in the Gulf *Ceratium bucephalum* and *longipes*, as shown by Tables III and IV, had their maxima below 30 m., at 40 to 50 m., where the conditions for photosynthesis could not be optimal, judged from the low oxygen content of the water. Only at Station 1331, where the limit between the surface layer and the deeper layers seemed to have been raised to a higher level, *C. bucephalum* had its maximum

as high up as 30 m. These two species were dominating the phytoplankton in the same area one month earlier, at the beginning of July, as was observed by the International Passamaquoddy Fisheries Commission, and have probably sunk down after their period of growth.

The two stations 1332 and 1333, taken in the eastern part of the Gulf, where the water according to Bigelow's observations flows into the Gulf from the south, were distinctly poorer than the others. Single specimens of rare oceanic species, like *Oxytoxum reticulatum* at Station 1333, prove the oceanic influence. But at the same time one may be allowed to conclude that the rich Ceratium-plankton of the Gulf has gotten its specific character in the Gulf itself by propagation of the species adapted to the local conditions, even if some of them may have floated in from outside.

Georges Bank

The plankton collections taken at the three stations on Georges Bank, 1334 to 1336, all show a marked difference from those of the inner Gulf,

Table IV

Ceratium longipes, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	—	20	—	—	—	—	—	—
10	—	—	20	—	—	—	—	—
20	—	20	—	—	—	—	40	—
30	—	20	80	—	20	—	20	—
40	60	60	380	—	—	40	—	—
50	—	280	200	—	—	—	20	—
75 (60)	—	80	40	—	200	20	—	—

but between them there is a great similarity. Table V shows as an example the observations from Station 1334.

The species dominating in the Gulf, the *Ceratium* species, *Gonyaulax orientalis, Pontosphæra Huxleyi, Coscinosira Œstrupi,* occurred also on the Bank, but in much smaller numbers. The plankton on the Bank was dominated by oceanic diatoms, first of all *Rhizosolenia alata* (Table VI), which at Station 1334 formed quite a dense population with a maximum of nearly 50,000 cells per liter.

Also, *Rhizosolenia Shrubsolei* and *Nitzschia seriata* occurred in fair numbers, up to 880 and 1440 per liter in nearly all the samples, and *Rhizosolenia styliformis* was found evenly distributed. The neritic

diatoms were less abundant; only *Leptocylindrus danicus* and *Thalassiothrix nitzschioides* were found in most of the samples in numbers up to 1400 and 1240 per liter, numbers which are quite inconsiderable in

TABLE V

Atlantis Station 1334, August 4, 1932

Depth, *meters*	1	9	17	26	35	43	52
Temperature, ° *C*	14.98	15.02	14.15	13.25	12.55	11.86	9.01
σ_t	24.08	24.04	24.37	24.58	24.73	24.88	25.41
Oxygen, *% of saturation*	103	103	102	101	101	99	95
Nitrate, *mg. N per cu. m*	20	27	24	40	45	60	100
	cells per liter	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
Coccolithus pelagicus	—	—	—	—	—	40	20
Pontosphæra Huxleyi	840	160	—	—	—	—	—
Dictyocha fibula	—	40	—	—	—	—	20
Actinocyclus Ehrenbergi	—	—	80	80	40	—	120
Coscinodiscus excentricus	—	—	40	80	—	—	40
Coscinosira Œstrupi	40	—	—	120	—	80	140
Guinardia flaccida	—	—	—	—	—	40	100
Leptocylindrus danicus	—	—	1240	320	480	880	160
Nitzschia delicatissima	—	160	240	400	200	480	40
N. seriata	120	680	560	1080	1160	1440	1060
Paralia sulcata	—	—	—	—	—	—	500
Pleurosigma Normani	—	40	200	80	40	40	60
Rhizosolenia alata	27880	29720	47920	49800	27360	30120	15860
R. Shrubsolei	560	120	400	880	760	200	460
R. styliformis	200	120	40	120	120	40	140
Skeletonema costatum	—	—	—	160	—	160	—
Thalassiothrix longissima	80	—	40	40	160	—	—
T. nitzschioides	280	440	1240	960	560	1200	860
Ceratium bucephalum	—	—	—	—	40	40	—
C. fusus	—	120	80	—	40	—	100
C. lineatum	—	40	—	—	80	—	60
C. longipes	—	—	—	—	40	—	20
C. tripos	40	—	—	—	—	—	20
Gonyaulax orientalis, cystæ	40	—	—	—	—	40	20
Favella denticulata	—	—	80	40	40	40	40
Helicostomella subulata	40	40	40	80	120	40	40

The following species were observed in small numbers:

Actinoptychus undulatus 10 m., 40; ***Chætoceros compressus*** 20 m., 320; ***Leptocylindrus minimus*** 20 m., 640; ***Rhizosolenia setigera*** 50 m., 80; ***Dinophysis acuminata*** 20 m., 40; ***Peridinium brevipes*** 50 m., 40; ***Pyrophacus horologium*** 40 m., 40.

Ptychocylis obtusa 50 m., 20; ***Lohmanniella spiralis*** 60 m., 20; ***Tintinnopsis*** sp. 60 m., 20.

comparison with the quantities of the same species often observed in coastal waters. *Guinardia flaccida*, which was found on the Bank by Bigelow in July, 1913 and 1914 as a dominant species, occurred also in

1932 at all three stations, but only in the samples from the deeper layers (Table XIV). It was most abundant at Station 1336 with a maximum of 340 per liter in 50 meters depth. The neritic tintinnid *Helicostomella subulata* was found evenly distributed at all three stations in numbers up to 120 per liter.

Very characteristic is the regular occurrence of some littoral diatoms,

TABLE VI

Rhizosolenia alata, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	160	40	1480	—	80	27880	3080	440
10	220	120	1780	120	20	29720	4320	1750
20	100	180	700	280	20	47920	23620	4040
30	60	160	200	220	20	49800	12720	1660
40	140	—	220	120	40	27360	7660	2720
50	—	60	20	20	20	30120	8220	1720
75 (60)	—	—	20	—	—	15860	5720	660

TABLE VII

Pleurosigma Normani, August 4–5, 1932

Depth	Stations		
	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*
1	—	—	20
10	40	20	25
20	200	20	120
30	80	40	60
40	40	120	160
50	40	40	60
60	60	40	220

Pleurosigma Normani, Paralia sulcata, and *Navicula distans.* Their distribution indicates with certainty turbulence of the waters by which material from a shallow bottom has been carried out to sea.

It is also remarkable that on the Bank all the species mentioned above were evenly distributed from the surface to the bottom (Tables VI and VII). Such a situation can only have been produced by a strong

vertical mixing of the waters, which is also indicated by the even distribution of the temperature and salinity. The diatoms show a slight maximum at all stations at the depths of 20 to 30 meters. Just at this depth the photosynthesis seemed to have its optimum in the Gulf and the same may have been the case on the Bank, and thereby the growth may have been stimulated. But the difference in numbers corresponds only to one or two cell divisions and thus the turbulence of the waters must have had a stronger influence on the vertical distribution of the diatoms than the factors regulating their growth. The higher temperature of the very surface layer, from 1 to 10 meters, seems to have been slightly unfavorable for the growth or perhaps for the floating power—because of the lower viscosity of the water—of most of the species, for instance *Leptocylindrus danicus* (Table VIII).

TABLE VIII

Leptocylindrus danicus, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	—	—	—	—	—	—	—	—
10	—	—	120	—	—	—	—	—
20	—	—	—	—	20	1240	1400	560
30	—	40	120	—	—	320	1340	440
40	—	—	360	—	—	480	580	420
50	—	—	—	—	—	880	300	380
75 (60)	—	—	—	—	—	160	640	240

A comparison between the Gulf and the Bank shows first of all that the same species are found over the whole area, but the quantitative and also the vertical distribution is different. The stratification in the inner Gulf has favoured the growth of the *Ceratia* and of *Pontosphæra* (Tables IX and X), accumulated by their active movements in the depths of optimal life conditions. On the other hand, the mixing of the waters on Georges Bank has brought into circulation sufficient quantities of nutrient salts to produce a strong growth of oceanic diatoms, *Rhizosolenia alata* and others, and the continued vertical mixing has caused their even distribution from surface to bottom. At the innermost station on the Bank, Station 1334, this population has reached a nearly maximal density for so large a species (Tables V and VI).

Neritic diatoms, which are as far as we know, the most rapid growers of the plankton of temperate seas, were quite scarce over the whole area, although single cells or chains were observed everywhere. The only locality where a slight accumulation of them could be observed, was Station 1331, where for some unknown reason the nitrate content was high right up to 30 meters depth, and just at this depth the diatoms had

TABLE IX

Ceratium fusus, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	80	20	—	—	—	—	—	40
10	220	40	20	20	60	120	40	20
20	420	240	500	40	40	80	100	40
30	620	360	280	—	—	—	—	20
40	—	20	—	—	—	40	—	—
50	—	—	—	—	—	—	—	—
75 (60)	—	—	—	—	—	100	—	—

TABLE X

Pontosphæra Huxleyi, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	240	260	2060	260	2720	840	40	—
10	3880	220	940	960	2120	120	80	80
20	15620	6640	80	860	10660	—	2680	—
30	2000	37200	—	580	4140	—	40	—
40	80	300	—	40	3060	—	20	20
50	—	—	—	40	80	—	—	—
75 (60)	—	—	—	—	—	—	—	60

increased (Table II). Evidently they require a higher concentration of nutrient salts than the surface layers of the Gulf of Maine contained at this season.

CULTURE EXPERIMENTS

As a contribution to the question of the requirements of nutrition of the plankton diatoms some culture experiments were made with the

same method as was used previously on the Norwegian coasts. A series of closed, glass-stoppered bottles containing sea water with known content of plankton were suspended in the sea, all under the same conditions of light and temperature, and with the addition of various nutrient salts. The increase of the diatoms was determined by counting. When the culture bottles were exposed only a few days, and the population in the bottles was not allowed to increase above the maximum density observed in the sea, about one million cells per liter, the cultures could be kept under quite normal conditions, and by use of a hand lens the cells or chains could be seen normally and evenly suspended in the water within the bottles.

The main question for the experiments arranged at Woods Hole was that of the possible influence of soluble iron compounds on the growth of the plankton diatoms. As it is now well known, most of the differences in the productivity of the various areas in the sea can be explained from the distribution of the nitrates and phosphates, acting as limiting factors for the growth of the plankton algæ according to Brandt's theory.

But we have also some indications that the quantities of soluble iron present in sea water may be insufficient to support a rapid growth, particularly of the neritic diatoms, and my working hypothesis is that the soluble iron compounds, washed out with "humus"-compounds from the shores, may give the explanation of the relative productivity of coastal waters in comparison with the open ocean.

In an experiment made on the Norwegian coast (Gran, 1931) I had found that the growth of the neritic diatom *Skeletonema costatum* was enormously stimulated by the addition of small quantities of garden soil extract, corresponding to 10 mg. soluble Fe per cu. m., to the oceanic water in which it was living. In this water, which contained sufficient quantities of nitrates and phosphates, no other nutrients gave, when added, any positive effect at all.

If the effect of the soil extract is caused by its content of soluble iron, it must be possible to replace the soil extract by other soluble iron compounds, as Carsten Olsen (1929) did in his experiments with *Lemna*, using ferri-citrate with positive results. Recently also Burk and his collaborators (Burk, Lineweaver, and Horner, 1932) have proved by exact experiments that the stimulating effect of soil extract on *Azotobacter* is caused by its content of soluble iron.

My previous experiments to replace the soil extract by other iron compounds were not very successful. Oxyhæmoglobin or ferrisaccharate gave no positive effect, and the effect of ferricitrate or ferrosulphate was too small in comparison with that of the soil extract, possibly because these compounds were too quickly decomposed or oxidized in sea water.

At Woods Hole Dr. Waksman kindly gave me an opportunity to test his synthetic ferri-ligno-proteid (Waksman and Iyer, 1932) which, according to his great experience, may be regarded as identical with the essential components of "humus" and which at least has the same biological and chemical reactions.

For the experiments clean, glass-stoppered bottles of 130 cc. inner volume were filled from the same container with sea water of known plankton content, and suspended in the harbor at a depth of 1.5 meters.

To the bottles was added:

(1) No addition (3 bottles).
(2) 1 cc. of a solution containing 0.1 per cent KNO_3 and 0.01 per cent $NaH_2\ PO_4$ (3 bottles).
(3) The same as (2) + 1 cc. of garden soil extract (1 liter garden soil, boiled in autoclave with 1 liter distilled water, then filtered, and the filtrate sterilized in autoclave) (3 bottles).
(4) The same as (2) + 0.1 cc. of a solution of 100 mg. ferri-ligno-protein in 500 grams water (3 bottles).

TABLE XI

Culture Experiment 1. Woods Hole, July 23–26. Temperature, 20°–22°. Surface water taken outside the harbor.

Diatoms	Before experiment	Without addition (1)	Nitrate and Phosphate (2)	Nitrate, Phosphate, and Soil (3)	Nitrate, Phosphate, and Ferri-ligno-protein (4)
	cells per cc.	*cells per cc.*	*cells per cc.*	*cells per cc.*	*cells per cc.*
Chætoceros compressus.....	2	1	243	310	415
Leptocylindrus danicus....	13	16	466	792	628
Nitzschia delicatissima.....	28	58	957	595	789

The first experiment was made on July 23 with surface water taken outside the harbor of Woods Hole. This water was rather rich in diatoms, and therefore the bottles were suspended for only three days, until July 26, and their content preserved with formalin for counting. The water originally contained per cubic centimeter 22 cells of *Rhizosolenia calcar avis,* 24 of *R. Shrubsolci,* 2 *Chætoceros compressus,* 13 *Leptocylindrus danicus,* 28 *Nitzschia delicatissima* and some other species in very small numbers. The two first-named species did not grow well in the bottles; for the other three the average number per cubic centimeter is given in Table XI.

The result is clear. Without addition of nutrients the diatoms had not propagated at all, but the addition of nitrate and phosphate, in

quantities found under optimal conditions in nature, was enough to stimulate them to a growth about equal to the maximal growth observed for plankton diatoms, corresponding to seven divisions in three days for *Chætoceros compressus* and five to six divisions for the other species. The extra addition of soil extract or of the ferri-ligno-protein had no marked effect. If the stimulating effect of the soil extract depends upon its iron content, the conclusion must be that the harbor water used for the experiment contained more than enough of soluble iron, while at the same time its content of nitrate or phosphate has been the limiting factor for further growth of the diatoms.

The experiment needed to be repeated with more oceanic water which, according to the theory, should contain less soluble iron if these compounds are renewed by outwash from shore. Therefore, on the last night of the cruise of the "Atlantis," the fifth of August, a large water sample was taken from the surface off Nantucket Lightship and a new experiment arranged the next day. The same plan as before was followed, except that to the bottles of series (4) was also added $MnCl_2$, corresponding to 10 mg. Mn per cubic meter. The water was rather poor in plankton. It contained six cells per cubic centimeter of *Rhizosolenia alata* and 0.4 cells per cubic centimeter of *Nitzschia seriata*. *Leptocylindrus danicus* must have been present to the number of 0.1 cells per cubic centimeter, although it was not found by counting a sample of 10 cc. The character of the plankton was rather similar to what was found at Station 1336 on Georges Bank.

The species reacted in different ways to the addition of the nutrients. *Rhizosolenia alata*, which is an oceanic species, had in five days without extra nutrition grown to make up a population of about the same density which was found at the innermost station on Georges Bank, Station 1334. With nitrate and phosphate it had grown very much more. The increase corresponds to six cell divisions in five days, which I have found to be the maximal growth rate for this species. The extra addition of soil extract or ferri-ligno-protein did not give any further effect.

The typical neritic species, *Leptocylindrus danicus*, reacted otherwise. With addition of nitrate and phosphate alone it increased from one to 130, which corresponds to seven divisions in five days, one cell division more than *Rhizosolenia*. But with soil extract it increased to a number ten to eleven times higher, and with Waksman's compound and $MnCl_2$ twenty-five fold. With soil extract it averaged ten to eleven cell divisions in five days, and with ferri-ligno-protein eleven to twelve divisions. The effect of the synthetic "humus" seems to be quite similar to that of the soil extract.

Nitzschia seriata also showed some effect of the soil extract and of

the ferri-ligno-protein, but less marked than *Leptocylindrus*. In its distribution it is not so strongly limited to the coastal waters as *Leptocylindrus*. It is one of the very few motile species among the plankton diatoms, which may perhaps give the explanation of its relatively good growth in the bottles without added nutrition.

We intended to continue the experiments and to use for comparison also ligno-protein without iron, but had no opportunity to get water of sufficiently oceanic character. A third experiment gave results parallel to those of the first, a maximal effect of nitrate and phosphate alone and no extra effect of the iron compounds.

The results published here are not conclusive and the experiments should be repeated when opportunity arises. The method is practicable where water with living plankton can be brought in from oceanic situations.

The question, whether the presence of soluble iron compounds in sufficient quantities can be a limiting factor to the growth of the plankton

TABLE XII

Culture Experiment 2. Woods Hole, August 6–11, 1932. Temperature 20–22°. Water taken off Nantucket Lightship on the night of August 5.

Diatoms	Before experiment	Without addition (1)	Nitrate, Phosphate (2)	Nitrate, Phosphate, and Soil (3)	Nitrate, Phosphate, ferriligno-protein, and Manganese (4)
	cells per cc.	*cells per cc.*	*cells per cc.*	*cells per cc.*	*cells per cc.*
Rhizosolenia alata	6	29	403	466	478
Nitzschia seriata	0.4	36	365	1109	769
Leptocylindrus danicus	0.1	1	13	150	335

The diatoms were quite normal in all cultures; in some bottles they had even formed auxospores. The figures represent averages of three parallel cultures.

diatoms, and particularly of the species with the most rapid growth rates, can not be solved, of course, until we also have more data about the distribution of the soluble iron compounds in the sea.

In collaboration with Dr. Waksman and Dr. Reuszer a preliminary experiment was also arranged to follow the relation of the marine bacteria to the plankton diatoms.

A series of bottles filled with sea water taken at the surface outside the harbor of Woods Hole were suspended in the sea in the same way as described above. To some of them was added nitrate and phosphate in the same relation as in Experiments 1 and 2, while the others were left without any addition of nutrients. Bottles were taken to be studied with regard to their content of plankton and bacteria after three, seven

and twelve days. The water was rather rich in plankton beforehand, and the number did not increase in the bottles without nitrate and phosphate. As shown in Table XIII, the plankton in the fertilized bottles had increased after three days to a maximal density. Most of the species were still in good condition and normally suspended in the water. After seven days most of the species had decreased in number, and dead cells formed lumps at the bottom of the bottles.

The bacteria increased and decreased with the diatoms, as Waksman and his collaborators report (in the paper following this one). The high temperature may have been one of the causes accelerating the decrease of the plankton after the maximum. Waksman's interesting idea of the correlation between the plankton and the bacteria can perhaps be studied favorably in experiments like this, in which the water is poor in plankton from the beginning and therefore the conditions in the bottles

TABLE XIII

Culture Experiment 4. August 18–30, 1932. Nitrate and phosphate as in Experiment 1 (2).

Diatoms	Before experiment	After 3 days	7 days	12 days
	cells per cc.	*cells per cc.*	*cells per cc.*	*cells per cc.*
Chætoceros compressus	16	95	18	57
Coscinosira Œstrupi	11	62	6	3
Leptocylindrus danicus	11	86	5	0
Nitzschia seriata	65	468	65	31
Rhizosolenia calcar avis	2	0	0	0
Rhizosolenia delicatula	36	32	7	0
Rhizosolenia fragilissima	21	84	37	0
Rhizosolenia setigera	6	6	21	27
Rhizosolenia Shrubsolei	1	0	0	0
Skeletonema costatum	(0)	139	3	9
Thalassiothrix nitzschioides	2	16	0	0
Other species	6	38	16	38
Sum	177	1026	178	165

can be kept normal for some days. When some of the diatoms begin to die too many unknown factors are introduced.

REMARKS ON THE SPECIES

Heterocontæ

Halosphæra viridis Schmitz is the only annual plant of the phytoplankton, as far as known. It has never been observed to occur in such an abundance as to be found in great numbers in the centrifuged water samples. Young specimens were found at Station 1330 at 20 meters

depth in a number of 340 per liter, and at Station 1333 it was observed in all samples from the surface to 50 meters, with a maximum of 140 per liter at 20 meters. Single specimens were observed at Stations 1332, 1334, and 1336.

Coccolithophorida

Coccolithus pelagicus Wallich was observed at the three stations in the inner part of the Gulf (1329–1331) and at Station 1336 on Georges Bank. Its maximum of frequency, 220 cells per liter, was found at Station 1330 near the surface, 1–10 meters. At Station 1336 it occurred in all samples from 20 meters downwards, with a maximum of 100 at 40 meters depth.

Pontosphæra Huxleyi Lohmann is the most common of all the Coccolithophorida. Although it is an oceanic species it can occur in enormous numbers, millions per liter, in coastal waters also, where the temperature is sufficiently high. As the table shows, it was found over the whole area, distinctly more abundant at the deeper stations in the Gulf than on Georges Bank, with a maximum of frequency of 37,000 per liter at Station 1330.

Silicoflagellata

The few species of this order have a wide distribution but never occur in abundance.

Dictyocha fibula Ehrenberg was observed at two stations in the Gulf and at three stations over Georges Bank, nowhere in higher numbers than 80 per liter.

Distephanus speculum Ehrenberg is a more northern species, which was observed only at Station 1331, together with the maximum of neritic diatoms, most abundant (120 per liter) at 30 meters depth, and at Station 1336.

Diatoms

In temperate and arctic waters the diatoms make up the bulk of the phytoplankton where the nutrient salts are present in sufficient quantities. Some of them, particularly the neritic species, as *Skeletonema costatum, Chætoceros debilis, C. compressus,* and others, *Leptocylindrus danicus, Thalassiosira Nordenskiældii,* are able to reproduce themselves under favorable conditions very quickly, with up to two cell divisions per day. Also some oceanic species, as *Rhizosolenia alata* and *R. semispina, Chætoceros decipiens, Nitzschia seriata,* and *N. delicatissima* can form very dense populations, but their propagation seems to be less rapid than that of the neritic ones mentioned above. Other species as, e.g., the large *Coscinodiscus,* can be very commonly distributed but never so abundant that more than a few specimens are found by centrifuging

50 cc. of sea water. A number of littoral species occur so regularly in the plankton that they have to be reckoned with as signs of turbulence of the water, although their number is quite insignificant in comparison with the true pelagic species. It is rather remarkable that only some few out of the great number of littoral species are found regularly in the plankton, as in our material *Actinoptychus undulatus, Navicula distans, Pleurosigma Normani,* and *Paralia sulcata.* They may perhaps even propagate to some extent in the floating condition.

Actinocyclus Ehrenbergii Ralfs. This species was observed at three stations in the central part of the Gulf (1329–1331) and at all stations on Georges Bank (1334–1336). Here it was found in all depths from 20 meters to the bottom in numbers of up to 300 per liter at Station 1336, at 30 meters depth. Its heavy, thick-walled cells are more often found in greater depths than in the surface layers, and it is in my opinion a characteristic representative of the " shade-flora " of Schimper (Karsten, 1905). Ostenfeld (1913) regarded it as a littoral, " tycho-pelagic " species.

Actinoptychus undulatus Ehrenberg was observed as single specimens at all stations on Georges Bank. It is without doubt a littoral species.

Corataulina Bergonii H. Peragallo. This neritic species had a small maximum in 20 to 30 meters depth at Station 1331 (160 to 180 per liter) together with the neritic *Chætoceros* species. Single specimens were also observed at Stations 1330, 1332, and 1336.

Chætoceros. Eleven species of this genus were observed. All of them except the oceanic species *Ch. atlanticus* occurred and had their maximum at Station 1331 (see Table II) at 30 to 40 meters depth as remains of a more abundant growth earlier in the season. At 30 meters the high nitrate content of the water may have caused some increase, but at 40 meters many of the species had formed resting spores and were evidently sinking. At Station 1329 the situation was similar but less pronounced. Here only the two most common species, *Ch. compressus* and *Ch. constrictus* occurred in small numbers (120 to 260 per liter), the former with resting spores.

On Georges Bank the oceanic species *Ch. atlanticus* and particularly *Ch. decipiens* occurred at the two outer stations, 1335 and 1336, *Ch. atlanticus* very scarce in a few samples (60–80 per liter), *Ch. decipiens* in all samples except at the surface, in numbers up to 340 per liter at 20–30 meters at Station 1335. Here also the neritic species *Ch. compressus* and *laciniosus* were found in small numbers, mostly with resting spores.

The species found in the material were: *Ch. affinis* Lauder, *Ch. at-*

lanticus Cleve, *Ch. borealis* Bailey, *Ch. compressus* Lauder, *Ch. constrictus* Gran, *Ch. convolutus* Castracane, *Ch. debilis* Cleve, *Ch. decipiens* Cleve, *Ch. diadema* Ehrenberg, *Ch. laciniosus* Schuett, *Ch. radicans* Schuett, *Ch. teres* Cleve.

Corethron hystrix Hensen occurred in small numbers at Stations 1330 and 1331, with a maximum of 180 per liter at 30 meters depth at Station 1331.

Coscinodiscus. The centrifuge method cannot give a good picture of the distribution of the larger species of this genus, as the quantity of water (50 cc.) is too small. *C. centralis* Ehrenberg was observed at Station 1330, *C. radiatus* Ehrenberg at Stations 1331 and 1336, but only as single specimens. *C. excentricus* Ehrenberg was found more regularly, at all stations except 1329 and 1332. It seemed to be more common over Georges Bank than in the deeper parts of the Gulf, with maximum records of 80 per liter at 30 meters depth at Stations 1334 and 1336.

Coscinosira Œstrupi Ostenfeld occurred regularly at all stations, less abundant on Georges Bank than in the inner part of the Gulf, and with a maximum of abundance 1800 per liter at Station 1331, at 30 meters depth, together with the maximum of the neritic *Chætoceros* species. This little species of which Hustedt (1927–30, p. 317) recently has given excellent figures and description, is one of the very few representatives of the plankton of the western side of the North Atlantic, which is lacking, or at least very rare along the European coasts.

Guinardia flaccida H. Peragallo was dominating in the plankton of Georges Bank in July, 1913 and 1914 (Bigelow, 1928). It occurred also in August 1932 at all stations on the Bank, but not in great numbers, and with its abundance increasing toward the bottom, as shown by Table XIV. *Guinardia* was not observed in the deeper parts of the Gulf.

Leptocylindrus danicus Cleve (see Table VIII) occurred regularly at all stations on Georges Bank from 20 meters to the bottom, with maximum at 20 meters depth at all three stations. It was also found in small numbers at Station 1331 together with the *Chætoceros*-plankton and in traces at Stations 1330 and 1333.

Leptocylindrus minimus Gran was observed in small numbers, up to 640 per liter at Stations 1334–1336 on Georges Bank, at 20–30 meters depth.

Navicula distans W. Smith was observed as single specimens at Stations 1329 and 1335. At Station 1336 on Georges Bank it was found in all samples from 10 meters to the bottom. It is a littoral species with thick cell walls.

Nitzschia closterium W. Smith. Single specimens at Stations 1329 and 1336.

Nitzschia delicatissima Cleve occurred regularly at all depths from 20 meters to the bottom at Stations 1334–1336 on Georges Bank, but in very small numbers, up to 480 per liter. At Stations 1329 and 1330 traces were observed.

Nitzschia seriata Cleve occurred as well in the inner part of the Gulf as on Georges Bank, with a maximum of 1440 per liter at Station 1334.

Paralia sulcata (Ehrenberg) is another littoral species, which was found as scattered chains at Stations 1332–1336 in the deeper layers of water.

Pleurosigma Normani Ralfs was observed in nearly every sample from Georges Bank, rather evenly distributed from the surface to the

TABLE XIV

Guinardia flaccida, August 4–5, 1932

Stations.....		1334	1335	1336
		cells per liter	*cells per liter*	*cells per liter*
Depth in meters	1	—	—	—
	10	—	—	—
	20	—	—	20
	30	—	—	160
	40	—	20	40
	50	40	140	300
	60	100	220	340

bottom, as a sign of the turbulence. In a sample of 50 cc., from one to eleven cells were counted (Table VII).

Rhizosolenia alata Brightwell was, in accordance with Bigelow's observations in previous years, the most common diatom at this season over the whole area and occurred at all stations, subordinate to the *Ceratia* in the stratified waters in the deeper part of the Gulf, but dominant on Georges Bank. Here it was distributed rather evenly from surface to bottom with a slight maximum at 20 to 30 meters depth. At Station 1334 at the inner side of the Bank the population was quite dense, with a maximum of nearly 50,000 cells per liter.

Rhizosolenia fragilissima Bergon (including *R. færoënsis* Ostenfeld), a neritic species which sometimes occurs in abundance, was observed only at Station 1331 at 30 meters depth in quite small numbers, together with the maximum of neritic *Chætoceros* species.

Rhizosolenia Shrubsolei Brightwell occurred together with *R. alata*, as it often does, but in much smaller numbers. On Georges Bank it was found evenly distributed from the surface to the bottom, in numbers up to 800–900 per liter. In the deeper part of the Gulf it was much less frequent and was limited to the layers between 20 and 40 meters depth.

Rhizosolenia styliformis Brightwell. This large and beautiful oceanic diatom had the same distribution as *Rh. Shrubsolei*, but occurred in smaller numbers. The highest frequency observed was 520 per liter at Station 1335 at 60 meters depth, but as a rule the number counted was only 200 per liter or less.

Rhizosolenia setigera Brightwell is a neritic species which was observed only in quite small numbers on Georges Bank, less than 100 per liter.

Skeletonema costatum (Greville). Single chains were observed at Stations 1331 and 1334.

Thalassiosira decipiens (Grunow) was observed quite rarely between 40 and 50 meters depth at the first two stations (1329–1330).

Thalassiosira gravida Cleve occurred at most of the stations but was quite scarce. The highest number observed was 140 per liter at Station 1329, 50 meters depth.

Thalassiothrix longissima Cleve and Grunow occurred rather evenly on Georges Bank, particularly at Station 1334, where it reached a maximum of 160 per liter at 40 meters. It is regarded as a subarctic oceanic species.

Thalassiothrix nitzschioides Grunow was only observed on Georges Bank but occurred there evenly distributed from surface to bottom in numbers from 200 to 1200 cells per liter.

Dinoflagellata

Ceratium. The species of this genus are by far the most important among the Dinoflagellata of the North Atlantic. They are oceanic, but are often found in greater abundance in semi-coastal areas as the North Sea, the Gulf of Maine, and the Gulf of St. Lawrence than in pure oceanic waters. Here they usually begin to dominate the phytoplankton as soon as the waters become stratified, after the spring growth of the diatoms has finished. They succeed each other according to their temperature requirements. *C. arcticum* and *C. longipes* dominate in the first part of the season, followed by *C. fusus* and *tripos*.

In the Gulf of Maine *C. fusus* was the dominating species in the beginning of August (Table IX) in the stratified waters. Its maximum of 500–600 per liter was found just at the depth where the values for

saturation with oxygen were maximal and the life conditions optimal for the phytoplankton, at 20 to 30 meters depth. Two other species, *C. longipes* and *C. bucephalum,* were also rather abundant at the same stations (Tables III and IV) but their maxima were found deeper down, 40–50 meters depth, evidently below the depth of the optimal conditions for photosynthesis, as the water at these depths was not saturated with oxygen. Other observations, from the investigations of the International Passamaquoddy Fisheries Commission, show that these two species were dominating not far from the localities where our stations were taken, one month earlier, in the beginning of July. One may conclude that they have sunk into deeper layers after their main growth period was ended and they were succeeded by *C. fusus.* From my experiences on European coasts it is astonishing to find *C. bucephalum* early in the season together with *C. longipes,* as we used to find its maximum in September, together with that of *C. furca* which I have not observed at all in the Gulf of Maine.

Besides these species *C. arcticum* was found in small numbers at Station 1329, from 20 to 50 meters depth.

Ceratium tripos (O. F. Mueller) occurred at the same stations as *C. fusus,* and at the same depths, but less abundantly. Its maximum was found at 20 meters at Station 1331, with 280 per liter.

Ceratium lineatum (Ehrenberg) seems to be more neritic than the other species. It was found at the surface at Station 1331 in the number of 240 per liter, and on Georges Bank it occurred at all stations, rather evenly distributed with regard to the depth, with a maximum of 150 per liter at Station 1336, at 10 meters depth.

Dinophysis acuminata (Claparède and Lachmann) was observed as single specimens at two stations, 1329 and 1334.

Dinophysis norvegica Claparède and Lachmann was also quite scarce. Single cells were observed at four stations, in seven samples.

Exuviælla baltica Lohmann occurred at all stations with its maximum near the surface. The highest number counted was 480 per liter at 10 meters depth at Station 1335. In coastal waters it can sometimes be found in much greater numbers.

Gonyaulax orientalis Lindemann. This species, described also by Miss Lebour (1925, p. 93) seems to be rather common. Rather few vegetative cells were found, but a number of globular thick-walled cysts, containing chromatophores and oil drops. Some of them were in cell division, and two naked cells escaped which developed into *Gonyaulax* cells. They were rather abundant in the inner part of the Gulf in depths from 10 to 30 meters, with a maximum of 1340 at Station 1331, but less

common on Georges Bank. The diameter of the cysts was from 45 to 64 μ, the thickness of their cell wall 1.5 μ.

Gonyaulax spinifera (Claparède and Lachmann) was rare in the deeper part of the Gulf. At Station 1329 80 cells per liter were found as a maximum.

Oxytoxum gracile Gran (1929, p. 45). Single specimens were observed at two stations (1330 and 1332).

Oxytoxum reticulatum (Stein). One specimen of this rare oceanic species was observed at Station 1332 at 20 meters depth.

Peridinium

The marine species of *Peridinium* (exclusive of *Minuscula* Lebour) are heterotrophic and rarely occur abundantly enough to be regularly found in the centrifuged water samples. Eight species were observed: *P. achromaticum* Levander, *P. conicum* Gran, *P. brevipes* Kofoid, *P. depressum* Bailey, *P. ovatum* Pouchet, *P. pellucidum* Bergh, *P. pyriforme* Paulsen, *P. Steinii* Joergensen. Of these only *P. Steinii* and *P. ovatum* were found in a maximum abundance of 100 per liter or more, both of them at Station 1331, where seven of the eight species were found.

Protoceratium reticulatum Bergh, one specimen at Station 1329.

Pyrophacus horologium Stein. This neritic and autotrophic species was found at all stations on Georges Bank, but always in small numbers, up to 60 per liter.

Infusoria ciliata

Acanthostomella norvegica (Daday) was observed in small numbers at four stations in the deeper part of the Gulf, with the maximum of 80 per liter, at Station 1329, 20 meters depth.

Favella denticulata (Ehrenberg), the most common tintinnid in the collections, was found at all stations, mostly in 20 to 30 meters depth, with a maximum of 320 per liter at Station 1329, 20 meters depth.

Helicostomella subulata (Ehrenberg) is a neritic species which was found exclusively on Georges Bank, and here at all stations, with the maximum of 220 per liter at Station 1335, 20 meters depth.

Labœa Lohmann. The species of *Labœa* are not found in the net samples, but some of them which live in symbiosis with small brown cells can at times occur rather abundantly in the centrifuged water samples, particularly in spring together with the neritic diatom plankton. In August they were very scarce. *L. conica* Lohmann and *L. strobila* Lohmann were observed at Station 1330 from 1 to 10 meters depth, *L. acuminata* Leegaard and *L. vestita* Leegaard at Station 1335.

Lohmanniella oviformis Leegaard was observed in small numbers at four stations, 1331–1333 and 1336. At Station 1333 it occurred from 10 to 30 meters depth with a maximum of 60 per liter at 30 meters.

Lohmanniella spiralis Leegaard. Single specimens were observed at Stations 1330 and 1334.

Mesodinium rubrum Lohmann was quite scarce at this season. At Station 1335 on Georges Bank about 100 per liter were found at 1 to 10 meters depth, at Station 1333 a single specimen at 20 meters.

Ptychocylis obtusa Brandt was observed at Stations 1329–1331 in the Gulf and on Georges Bank at Stations 1334 and 1335, with a maximum of 80 per liter at Station 1331, 30 meters depth. A single specimen of *Tintinnopsis campanula* (Ehrenberg) was found at Station 1335.

Stenoscmella sp. occurred on Georges Bank and at Stations 1332–1333 with a maximum of 100 per liter at Station 1335, 20 meters depth.

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STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

III. Bacteriological Investigations of the Sea Water and Marine Bottoms

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In former expeditions undertaken for the study of marine life, the occurrence, abundance, and activities of the bacteria were either left out entirely, or were based upon the examination of samples of water brought back many days and frequently months after they were collected. The bacteriological activities in the sea bottom were frequently completely overlooked. In view of the fact that the total numbers of the bacteria as well as the relative abundance of specific forms will change rapidly soon after the samples of water and mud are exposed to surface temperature, due either to a change in environmental conditions or to a change in food supply, it is quite essential that the collected samples should be subjected to immediate investigation. As a result of these considerations, the laboratory on the "Atlantis," during the cruise made in August, 1932, was so outfitted that it was possible to carry out the bacteriological studies within a few minutes or at most hours, after the samples of water, bottom mud, or sand were brought up from their respective depths.

Because of the extensive hydrographic and biological investigations which have been carried on in the Gulf of Maine, this region is particularly well adapted to a study of the relative abundance and activities of marine bacteria. Such a study was further aided by the fact that other biological and chemical investigations were carried on simultaneously on this cruise. The locations of the various stations at which samples were taken during this cruise are described in the first paper of this series.[2] Of the eight stations studied, five were in the Gulf of Maine proper and the remaining three in the shallow waters over Georges

[1] Contribution No. 10 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station.

[2] Rakestraw, Norris W., 1933. Chemistry of the Waters of the Gulf of Maine in August, 1932. *Biol. Bull.*, **64**: 149.

Bank. A detailed description of the methods used in these investigations will be presented later, when the bacteriological results of this expedition will also be compared with those obtained from waters and sea bottoms near shore. Only a brief summary of the investigations carried out on and in connection with this cruise of the "Atlantis" will be reported here. An attempt will be made to interpret the results of these investigations in the light of the chemical conditions of the waters and nature of the marine bottom, as well as to correlate the results of the bacteriological investigations with the results of the chemical and planktonological studies made by the other investigators on this cruise.

Abundance of Bacteria in Sea Water and in the Sea Bottom

The relative numbers of bacteria in the sea are usually determined by means of the plate method. This method has numerous limitations and can at best give only a very approximate idea of the actual abundance of the bacteria, both in the water and in the sea bottom. Although new methods have been introduced recently, consisting in direct staining of the bacteria in the water, after they have been concentrated by filtration or centrifuging, these methods are still in an experimental stage and were not used on this expedition. If the various limitations of the plate method are kept in mind and if broad generalizations are avoided, the results obtained even by this procedure can be made to throw considerable light upon the relative abundance and nature of the bacterial population of the sea.

The numbers of bacteria were determined in the water from the different stations at three different depths, namely, the surface, 50 meters, and the bottom. At one station, water from two additional depths of 30 and 100 meters was also investigated. The surface samples were taken with ordinary sterilized and evacuated glass tubes provided with a sealed glass tip which was broken about one foot below the surface of the water. Deeper samples were taken in heavier-walled glass tubes having a specially constricted neck to prevent the rubber stopper being forced into the tube by the pressure of the water. The inlet tube in this case was made of a capillary glass tube having a 1 mm. bore. Tubes of this type were used successfully to a depth of 330 meters but the lower limit of their usefulness has not yet been determined. Mud samples were taken by means of a sampler consisting essentially of a brass tube carrying a weight sufficient to force it some distance into the mud when the bottom is reached. Into the brass tube is fitted a glass tube having a check valve attached to its upper end. This allows water to stream through the tube during its descent. While raising the sampler the

valve closes and prevents any downward pressure upon the mud core within. By sterilizing the glass tube before use it is possible to eliminate any bacterial contamination of the mud sample other than the contamination of the walls of the glass tube by the water passing through it while descending. This may be easily eliminated by taking the sample to be studied from the center of the core and discarding the surface which has been in contact with the glass tube.

The water was plated undiluted and diluted 10 times, while the mud was plated in dilutions of 1:10, 1:100 and 1:1000. Previous experiments, which will be reported elsewhere, have shown that the following medium is satisfactory for the development of marine bacteria, capable of growing on the ordinary plate:

Agar	15 grams
Glucose	1 gram
Peptone	1 gram
K_2HPO_4	0.05 gram
Sea water	1000 cc.

TABLE I

Abundance of bacteria in sea water. Total numbers in 1 cc. of water.

Station Number		1329	1330	1331	1332	1333	1334	1335	1336
Total depth of water, *meters*		256	207	230	346	230	71	74	64
Depth of sample, *meters*	Surface	10	3	4	2	380	16	92	6
	30			0					
	50	1	1	2	1	2			
	100			1					
	Bottom	6	0	1	1	3	25	41	27

The plates were incubated at air temperature and the colonies counted at the end of 7 and 10 days. All the samples of water and mud were plated out within a few minutes after being taken.

The total numbers of bacteria in the water, as determined by the plate method, are given in Table I. With respect to their bacterial content, the stations may be divided into two groups, the first containing the five deep water stations and the second the three shallow stations. With the exception of the surface water of Station 1333, the bacteria in the water of the first group, namely, Stations 1329 to 1333 inclusive, were uniformly so extremely low in number, that it is difficult to draw reliable conclusions in regard to their distribution. It would seem, however, that the bacteria were slightly more numerous at the surface than in the lower depths of water. There was no appreciable variation between

the different other depths sampled, either at a single station or between different stations. Neither was there any appreciable variation in the bacterial content of the surface water of the different stations of this group. The very high content of the surface water of Station 1333 was probably due to the sample inadvertently being taken near floating seaweed or the introduction of some other contamination. The water samples from the lower levels of this station resembled those from the other deep water stations in their bacterial content.

The bacteria in the water of the second group of stations were uniformly much more numerous than in the water from the deeper stations. In view of the other results to be reported below, this may be ascribed to the higher phytoplankton content of this water. In two of the three stations the bottom water samples were considerably higher in bacteria than the surface samples. One must keep in mind the fact that the bottom of the last group of stations was sandy, while the bottom of the first four stations was mud. To what extent the deposits at the bottom of the ocean directly affect the bacterial content of the water for any very great distance above, except in turbulent waters, still remains to be determined. Because of the stray of the sampling cable from the perpendicular and the rise and fall of the ship with the waves, it is not possible to secure water samples with the apparatus used nearer than 2 or 3 meters from the bottom. The results, therefore, cannot be taken as indicating the bacterial content of the layer of water in contact with the mud bottom. It is possible to secure water samples nearer the bottom at shallow stations, as in the case of the second group. This, together with the fact that the water at these stations is rather turbulent, may indicate the transfer of sufficient nutrient material from the bottom deposit to the water immediately above to support the greater number of bacteria found in these waters.

The more uniform distribution of the bacteria in the water at the shallower stations is also correlated with the even distribution of the diatoms. This is probably due to the stronger vertical mixing of the waters, as shown by the even distribution of temperature and salinity.

The results reported above agree with those of various other investigators in indicating that the numbers of bacteria in sea water are relatively low and quite uniform in distribution. In an effort to secure further information concerning the presence of bacteria in sea water, the association of these organisms with the plankton forms was investigated. By towing with plankton nets samples of both zoöplankton and phytoplankton were collected. A number 20 silk net was used, which left out the nannoplankton. The zoöplankton consisted largely of copepods, while the phytoplankton was made up almost entirely of

diatoms with a few *Ceratium* cells present. This material was collected in the glass jar at the bottom of the net and portions of it immediately plated out. The numbers of bacteria in the various tow waters are given in Table II.

The water containing the diatom plankton is extremely rich in bacteria. It was not possible to make determinations of the actual quantity of plankton present in the samples. Apparently the zoöplankton has fewer bacteria associated with it than the phytoplankton. Dr. Gran estimated that the sample obtained from Station 1336 contained 11,900 planktonic organisms per liter of tow water, of which 11,100 were diatoms. It is of considerable interest to compare the relative numbers of diatoms in the tow and in the sea water at this station with the numbers of bacteria in the tow water and in the sea water. In the case of the zoöplankton (Station 1331), there were 225 times as many bacteria in 1 cc. of the tow as in 1 cc. of the corresponding water, thus giving a ratio of the bacteria in the tow to the bacteria in the water of 225:1.

TABLE II

Abundance of bacteria in tow water. Numbers in 1 cc. of tow.

Station No.	Type of plankton	Total bacteria	Agar-decomposing bacteria	Percentage agar-decomposing bacteria
1331	Zoöplankton	890	0	0
1335	Diatom plankton	36,700	2,100	5.7
1336	Diatom plankton	37,500	2,500	6.7

In the case of the diatom plankton, the ratio between the bacteria in the tow and the bacteria in the water (taking the average of the surface and bottom samples) was 500:1 and 2,270:1, for Stations 1335 and 1336 respectively. It is important to note that Dr. Gran found that the ratio for the respective numbers of diatoms in the tow and in the water was about 1,667:1. This indicates that a definite parallelism exists between the bacteria and the diatoms in the water and in the plankton. The actual existence of such relations is substantiated by the fact that the bacteria from the diatom tow showed a considerable abundance of agar-liquefying organisms. While one encounters only infrequently such organisms in the free water, usually only very near shore, and in the mud, they were found to make up 5.7 and 6.7 per cent of the total bacterial flora in the plankton tow, as determined by the agar plate method. It is interesting to note that the bacteria found in the copepod-plankton did not include any agar-liquefying forms.

It is quite possible, therefore, that bacteria exist only to a very

limited extent in the free water of the sea, but are largely attached to the plankton organisms, living upon the dead members of the plankton or upon the excretion products of the cells, upon the cell membranes, especially the mucilaginous substances secreted by certain algæ, etc. One need not, of course, imagine that if such an associative growth exists, the numbers of higher plankton forms and bacteria will always be parallel. One can readily imagine that the bacterial maximum may be attained after the diatom and copepod maximum. This has been brought out in an experiment carried out in cooperation with Dr. Gran and described by him elsewhere, in which the numbers of diatoms and of bacteria were determined simultaneously in artificial cultures of diatoms in sea water (Table III). The rise in numbers of both groups of organisms appears to have been directly related. The increase in bacterial numbers was much greater proportionately than that of the diatoms. When the multiplication of the latter reached a maximum,

TABLE III

Comparative numbers of diatoms and bacteria in diatom cultures. Numbers in 1 cc. of culture water.

	Original water	After 3 days growth	After 7 days growth	After 12 days growth
Diatoms.....	177	1,026	178	165
Bacteria.....	346	20,600	22,800	13,900

the numbers of bacteria continued to rise further, while the numbers of diatoms began to fall. This may be noted at the 7-day period when the diatoms had dropped to their original level, while the bacterial numbers were still increasing. This is to be expected since the death of the diatoms provides a source of food for the saprophytic bacteria. The numbers of bacteria dropped slowly and, within the course of the experiment, did not fall to the level of the original water as did the diatoms, indicating a greater resistance of bacteria to the artificial culture conditions.

Because of the large numbers of bacteria capable of decomposing agar found associated with the diatoms of Stations 1335 and 1336, it was of interest to follow their behavior in this experiment. In the original water the number of agar-decomposing bacteria was of the order of 2 or 3 cells per cubic centimeter. At the end of the 3-day culture period this figure had risen to 50 cells per cubic centimeter. At none of the later periods did any agar-decomposing bacteria appear on

TABLE IV

Abundance of bacteria in the sea bottom. Numbers in 1 gram of mud or sand, dry basis.

Station Number	1329	1330	1331	1332	1335	1336
Surface	5,400	4,700	830	3,000	1,280	670
10 cm	1,720	1,700	140	290		

the plates. The disappearance of these bacteria may be ascribed to their overgrowth by other forms better adapted to the cultural conditions. This is shown by the fact that while the colonies on the plates from the original water indicated the presence of a considerable variety of bacterial forms, in the last plating from the cultures only one or two types remained.

The terrigenous bottom deposits of the Gulf of Maine contain varying amounts of organic matter originating partly from the organisms

TABLE V

Presence of Anærobic Bacteria in the Marine Bottom

Medium		Solution				Agar			
Dilution		10		100		10		100	
Station No.	Depth of mud	1	2	1	2	1	2	1	2
1329	0–30 cm.	+	+	+	0	5*	2*	0*	2*
	30–60 cm.	+	0	+	0	12	2	4	8
	60–90 cm.	+	0	0	0	+	+	0	0
1330	0–30 cm.	+	+	+	0	30	18	10	8
1331	0–30 cm.	0	0	0	0	?	?	0	0

\+ Indicates growth and gas formation.
* Figures = numbers of colonies in the agar shake-tube.

living in the sea bottom and sinking from the water above, but probably more largely from the organic matter present in a partly decomposed state in the sediments brought in from land. These forms of organic matter furnish the energy for the growth of the bacteria in the marine bottom. The total organic matter content in the mud and sand bottoms of the stations investigated is reported later, while a chemical study of the organic matter in sea bottoms or marine humus is reserved for another publication.

Mud samples for bacteriological investigation were obtained from Stations 1329 to 1332. The bottom at Station 1333 was made up of

pebbles and no sample could be secured. The sand bottom of Stations 1334 to 1336 could not be sampled by the procedure outlined above because of the sand slipping from the tube as it was withdrawn from the bottom. However, at two of these stations sand samples were obtained by means of a Petersen grab. The numbers of bacteria found in these samples, both at the surface of the sample and at a depth of 10 cm., in the case of the mud bottoms, are given in Table IV.

TABLE VI

*Numbers of bacteria in sea water and in mud, as shown by the dilution method, using gelatin as the medium for bacterial development.**

Station	Depth†	Dilution	Water	Mud
		cc.		
1329	Surface	0.1		++
1329	Surface	0.01		++
1329	Surface	0.001		++
1329	10	0.1		++
1329	10	0.001		+
1330	Surface	0.01		++
1330	Surface	0.001		+
1330	Surface	0.0001		0
1330	10	0.01		++
1330	10	0.001		0
1331	Surface	0.01		++
1331	Surface	0.001		++
1331	Surface	0.1	+	
1331	Surface	0.01	0	
1331	30	0.1	0	
1331	30	0.01	0	
1331	50	0.1	+	
1331	50	0.01	0	
1331	100	0.1	+	
1331	100	0.01	+	
1331	215	0.1	0	

* + indicates growth after 3 weeks incubation; ++ indicates very good growth.

† Depth in water, is, in meters, from surface of water; depth in mud is, in centimeters, from surface of mud.

The bacterial numbers in the surface layer of the mud were found to vary from 830 to 5400 per gram of dry mud. These figures for the mud may be compared with the values of 670 and 1280 per gram of dry sand at Stations 1335 and 1336. The two samples of mud (1329 and 1330) which contain more organic matter contain also more bacteria; variations which occur in the different mud samples may be ascribed to differences in organic matter content or to physical variations.

The total number of organisms decreases rapidly with the depth of

mud. Thus at Station 1329, at a depth of 10 cm., the number of bacteria is only 1,720 as compared to 5,400 at the surface. The decrease is even more noticeable in the mud of Station 1332. The bacteria do not disappear rapidly, however, below the surface layer of the mud, but extend to considerable depths, as shown by the data for the presence of anærobic organisms (Table V). These bacteria were determined by the dilution method, using liquid medium kept under anærobic conditions, and by the shake-tube method, using agar medium. The results indicate the widespread occurrence of anærobic bacteria near the surface of the mud and their presence to depths as great as 90 cm. below the surface.

There is no doubt that the numbers of bacteria found in sea water and in the sea bottom by the plate method represent only a fraction of the total numbers of bacteria. This is due to the limitations of the method, whereby only a part of the bacterial population develops on the plate into colonies. In order to check this, several samples of water and mud were diluted and 1 cc. portions of the various dilutions added to sterile portions of 10 per cent gelatin in sea water. The results presented in Table VI show that whereas the plate method gave only 1 to 4 cells in 1 cc. of water from Station 1331, the dilution method with gelatin as a medium gave positive growth in some instances with 0.1 cc. of water and in some even with 0.01 cc. of water, indicating that at least 10 to 100 bacterial cells were present in 1 cc. of the water. In the case of the mud samples, abundant positive growth was obtained in most instances with 0.001 gram.

Results of bacterial investigation of the water and bottom samples of the Barents sea, recently reported by Butkewitch,[3] also bring out the fact that the dilution method may give 10 to 100 times as many bacteria as the plate method. The direct microscopic method gives even larger numbers, usually a thousand times greater than those obtained by the plate method. This difference is due to the fact that not only living but also dead bacteria are counted by the microscope, while many bacteria unable to develop on artificial media are seen with the microscope.

Occurrence of Specific Groups of Bacteria in the Sea

A number of bacteria active in certain specific processes, which are no doubt of considerable importance in the metabolism of the sea, were demonstrated both in the sea water and in the sea bottom. It is sufficient to mention here the presence of ærobic and anærobic nitrogen-fixing bacteria, of nitrifying bacteria, of agar-liquefying and cellulose-decom-

[3] Butkewitch, W. C. Method of Bacteriological Investigation and Certain Data on the Distribution of Bacteria in the Water and Bottom of Barents Sea. Trans. Russian Oceanogr. Inst., **2**: No. 2, 1932.

posing bacteria, of nitrate-reducing bacteria, of various spore-forming and non-spore-forming bacteria, and of a number of heterotrophic ærobic as well as anærobic bacteria. Some of these organisms will be discussed in detail later, while others need only be mentioned here in passing.

At least three types of cellulose-decomposing bacteria were demonstrated to be present in the sea. They belong largely to the groups described by Winogradsky as *Cytophaga, Cellvibrio,* and *Cellfalcicula.* They produce yellow and orange pigments or no pigment at all. They were found in the water from Station 1329 and to a less extent in the mud of the same station, but they were especially abundant in the tow water collected at Station 1336. This phenomenon may suggest the probability that these organisms, as well as the agar-liquefying bacteria, also present abundantly in the diatom tow, as pointed out previously, are not found to any great extent in the free water or in the mud, but develop in close connection with the growth of the plankton organisms, especially the phytoplankton; one would expect that the latter would offer a logical substrate for the growth of such bacteria. Some of these bacteria were able to attack both cellulose and agar, while others attacked cellulose alone and still others grew upon agar alone.

The agar-liquefying bacteria are represented in the sea by a number of different groups. They produce yellow, pink, or brownish pigments or no pigment at all. Most of them are ærobic, while some are anærobic. A detailed description of these organisms will be published in a later study. Chitin-decomposing bacteria have also been demonstrated in the sea water but these organisms have as yet been insufficiently studied.

It is commonly assumed that the sea harbors very few spore-forming bacteria. This may be true of the water itself; the sea bottom, however, especially the mud bottom, was found to harbor large numbers of such bacteria. The spore-forming bacteria could best be demonstrated in the nitrogen-free media inoculated with various dilutions of the mud samples. Many of the spores were found to have the typical appearance of *Clostridium,* so that one came to expect the development of these organisms in the nitrogen-free media inoculated with mud. This organism was accompanied by other bacteria, some of which were spore-forming. Many spore-forming bacteria were found abundantly in the calcium acetate-sodium nitrate medium used for the study of nitrate-reducing bacteria.

Anærobic bacteria were demonstrated by the use of liquid and solid media sealed with vaseline. Gas formation in the liquid media and colony development in the shake-tubes were taken as evidence of the development of these organisms. The ordinary peptone-glucose medium

was used for this purpose. The cultures were inoculated with material from the mud profiles (Table V). The formation of colonies in the anærobic cultures brought out the fact that at least several hundred anærobic cells are present in each gram of moist mud, even to a depth of 60 cm. of mud.

Occurrence of Nitrogen-fixing Bacteria in the Gulf of Maine

The problem of nitrogen-fixation in the sea has always aroused considerable interest due to the importance of this phenomenon in marine metabolism. If it is true, as some oceanographers assume, that the sea produces as much plant and animal life as a similar area of land, the amount of nitrogen required for the synthesis of these numerous forms of life is quite enormous, since the nitrogen content of the various representatives of marine life varies from 0.7 per cent in the case of certain algæ, such as *Fucus,* to 10 per cent in the case of various animals which range from the lowly copepods to the large fish and mammals.

By the use of silica-gel plates and liquid media containing a layer of sand, the presence in the sea of the two important groups of non-symbiotic nitrogen-fixing bacteria, namely the aerobic *Azotobacter* and the anærobic *Clostridium,* was definitely demonstrated. The occurrence of organisms in the sea water and sea bottom of the Gulf of Maine, capable of growing on nitrogen-free liquid media, is brought out in Table VII. The nitrogen-fixing organisms were found both in the surface waters and in the marine bottom. Samples of water taken from Station 1331 at depths of 30, 100, and 215 meters gave largely negative results.

A detailed description of the methods used in this study as well as the nature of the organisms found and their nitrogen-fixing capacity will be reported in another publication. It suffices here to say that a nitrogen-free medium containing a carbon source (glucose, mannitol, salts of organic acids), a source of phosphate and organic iron was used. In the case of the plates, this medium was added to silica-gel prepared in large Petri dishes (15 cm. in diameter); it was then subjected to dialysis in tap water, and the plates finally soaked for a few minutes in boiled sea water. Each plate contained 2 grams of carbon source. The liquid medium was placed in large test tubes with a layer of sand on the bottom and sterilized.

The silica gel plate inoculated with mud from Station 1329 gave a

TABLE VII

Distribution of bacteria capable of growing on nitrogen-free media in ocean waters and marine bottoms

Station No.	Depth†	Dilution in cc. or grams	Sea Water						Marine Bottom					
			Mannitol			Glucose			Mannitol			Glucose		
			Growth*	Gas	Organisms‡	Growth	Gas	Organisms‡	Growth	Gas	Organisms‡	Growth	Gas	Organisms‡
1329	Surface	1.0	T, P	0	Bac.	P	0	Rods	–	–	—	–	–	–
1329	Surface	0.1	T	0	Bac.	T, P	0	–	T, P	+	Bac.	T	+	Bac.
1329	Surface	0.01	0	0	0	0	0	0	P	+	Bac.	T	–	–
1330	Surface	1.0	–	–	–	–	–	–	T, P	+	l.r.	–	+	sm.r.
1330	Surface	0.1	–	–	–	–	–	–	T	+	–	P, T	+	–
1331	Surface	1.0	T, P	–	sm.r.	T	0	sm.r.	–	–	–	–	–	–
1331	Surface	0.1	–	–	–				T	+	Bac.	T	+	Bac.
1331	Surface	0.01	–	–	–				T	+	Bac.	T	+	Bac.
1331	30	1.0	–	–	–	–	–	–						
1331	50	1.0	T, P	0	0	T	0	–						
1331	100	1.0	P	0	0	T	0	–						
1331	215	1.0	0	0	0	T	0	–						
1332	Surface	0.1	–	–	–	–	–	–	P	+	Bac.	T	+	l.r.
1332	10	0.1	–	–	–	–	–	–	–	–	–	T	+	–
1333	Surface	0.1	T, P	0	–	T, P	0	–	–	–	–			
1333	Deep	0.1	–	–	–	T, P	0	sm.r.						
1334	Surface	0.1	T	0	–	T	0	–	–	–	–			
1334	Deep water	0.1	T, P	0	sm.r.	–	–	–	–	–	–			
1335	Surface	1.0							T, P	+	sm.r.	T, P	+	l.r.
1336	Surface	1.0							P	–	sm.r.	P	+	sm.r.

* P = pellicle formation, T = turbidity.

† Depth in water, in meters, is from surface of water; depth in mud, in centimeters, is taken from surface of mud.

‡ Bac. = spore-forming rods; sm.r. = small rods; l.r. = large rods.

typical development of *Azotobacter chroococcum*. This organism was readily isolated and grown in pure culture. On nitrogen-free sea water media, with glucose or mannitol as a source of energy, it fixed four to six milligrams of nitrogen for one gram of carbohydrate in 21 days.

Occurrence in the Gulf of Maine of Bacteria Capable of Oxidizing Ammonium Salts to Nitrites

If there is any one group of bacteria that has attracted the greatest attention of oceanographers and of bacteriologists alike, it is the group of nitrifying bacteria, or those organisms which produce nitrites from ammonium salts and nitrates from nitrites. A review of the various theories proposed to explain the occurrence of nitrite and nitrate in the ocean would in itself require considerable space. It suffices here to bring out only the results of the "Atlantis" expedition, to enable one to draw some very definite conclusions. It is important to emphasize in this connection that negative results do not necessarily mean the absence of the specific organisms but merely that the possibility exists that the medium used for demonstrating these organisms and the conditions essential for their development were not particularly favorable. Positive results are, however, much more important.

Of the two processes leading to the formation of nitrate, the first one, namely the oxidation of ammonium salts to nitrite, is usually considered to be more specific and more important, hence this process was more emphasized during this cruise.

Preliminary investigations of the water taken near shore brought out the fact that nitrite formation can take place in a sea water medium if proper conditions are provided. Three methods were used for this purpose: (1) Sand and nutrient salts—$CaCO_3$, $(NH_4)_2SO_4$, K_2HPO_4—were added, in proper concentration to flasks and these were sterilized; the fresh sea water taken from the various stations and at the different depths was introduced directly into these flasks. (2) In another set of flasks sand taken from Georges Bank was used in a fresh state and water taken from some of the stations was added. (3) In a third experiment, sea water, sand, and chemicals were placed in flasks, sterilized, and inoculated with mud from the various stations. The results obtained on the "Atlantis" cruise are presented in Table VIII. These results prove definitely that nitrite-forming bacteria are completely absent in the free sea water or are present there only in very limited numbers, while the sea bottom contains an abundance of such organisms. In the case of the mud, the presence of nitrifying bacteria was demonstrated at quite appreciable depths. This tends to demonstrate that nitrite formation and probably also nitrate formation take

place in the sea bottom. The nitrite and nitrate ions will then diffuse into the water and will be gradually brought upwards by ocean currents, etc. Near the surface of the water, where active photosynthesis takes place, the nitrates tend to disappear due to the fact that they are rapidly consumed by the phytoplankton organisms.

Occurrence in the Sea of Bacteria Capable of Reducing Nitrates to Nitrites and to Atmospheric Nitrogen

The presence in the sea of bacteria capable of reducing nitrates has attracted considerable attention from oceanographers, largely as a result of the hypothesis proposed by Brandt,[4] who attempted to base the whole

Table VIII

Presence of nitrifying bacteria in sea water and sea bottom

Nitrite-forming bacteria in water			Nitrite bacteria in sea bottom		
Station No.	Depth of water	Nitrite formation in 20 days	Station No.	Depth of mud	Nitrite in 11 days
	meters			*cm.*	
1329	Surface	−	1329	0–30	+++
1329	Bottom	−	1329	30–60	++
1330	Bottom	−	1329	60–90	trace
1331	Surface	−	1330	0–30	++
1331	30	trace	1330	30–60	trace
1331	50	+	1330	60–90	trace
1331	100	−	1331	0–35	trace
1331	215	+	1336	Sand	+
1331	Bottom	−			
1332	Surface	+			
1332	50	+			
1332	100	+			
1333	Surface	trace			
1334	Surface	−			
1334	Bottom	trace			

system of marine metabolism on the occurrence and activities of these organisms. Preliminary studies in this laboratory have shown that when a liquid medium containing Ca-acetate, $NaNO_3$, and a layer of sand is inoculated with shore water or with mud, active nitrite formation will take place, accompanied by abundant gas evolution. When this medium was inoculated with mud, even in a dilution of 1:1000, the nitrate disappeared completely within 5 days. In other words, both the water and the mud close to the shore contain an abundance of organisms

[4] Brandt, K. Ueber den Stoffwechsel im Meere. Wiss. Meeresunters. Kiel, N. F., **4**: 213–230, 1899; **6**: 23–79, 1902; **18**: 185–429, 1916–1920; **19**: 251–253, 1919.

capable of reducing nitrates to nitrites, as well as of reducing the latter completely to atmospheric nitrogen.

For the investigations carried out on the "Atlantis" cruise, a medium similar to the above and containing 10 grams Ca-acetate, 1 gram $NaNO_3$, 0.5 gram K_2HPO_4, and a trace of iron per liter of sea water

TABLE IX

Relative abundance of nitrate-reducing bacteria in sea water

Station number	Depth of water	Dilution of water	Growth of bacteria	Microscopic examination	Gas evolution	Nitrite formation
	meters	*cc.*				
1329	Surface	1.0	Turbid	Large round cells and curved rods	–	+++++
1329	Surface	0.1	–	–	–	–
1329	Surface	0.01	–	–	–	–
1331	Surface	1.0	Turbid	Minute coccobacilli	–	+++++
1331	Surface	0.1	Slight turbidity	Minute coccobacilli	–	+++++
1331	Surface	0.01	–	–	–	–
1331	30	1.0	Turbid	Cocci and bacilli	–	+++++
1331	30	0.1	Turbid	Cocci and bacilli	–	+++++
1331	30	0.01	–	–	–	–
1331	50	1.0	Turbid	Cocci and bacilli	–	+++++
1331	50	0.1	–	–	–	–
1331	100	1.0	–	–	–	–
1331	100	0.1	–	–	–	–
1331	215	1.0	Slight turbidity	Long rods and cocci	–	+++++
1331	215	0.1	–	–	–	–
1333	Surface	0.1	Turbid	Small coccobacilli	–	+++++
1333	Surface	0.01	Turbid	Small coccobacilli	–	+++++
1333	Surface	0.001	Turbid	Small coccobacilli	–	+++++
1333	Bottom water	0.1	–	–	–	–
1333	Bottom water	0.01	–	–	–	–
1334	Surface water	0.1	–	–	–	–
1334	Surface water	0.01	–	–	–	–
1334	Deep water	0.1	–	–	–	+++++
1334	Deep water	0.01	–	–	–	–

was placed, in 25 cc. portions, in large test tubes containing a layer of sand. The medium was sterilized at 15 lbs. for 15 minutes. The tubes were inoculated immediately after the samples were taken, using various dilutions of water and mud. After two weeks incubation at air temperature, the cultures were examined microscopically and tested for nitrite (Tables IX, X).

Of all the cultures inoculated with water or with mud, only one gave positive gas evolution, namely, the one inoculated with 0.1 gram of mud from Station 1329. Positive growth of bacteria was always accompanied by a reduction of the nitrate to nitrite. This experiment tends to prove that the waters and the mud in the Gulf of Maine are able to reduce nitrates only to nitrites, but not to atmospheric nitrogen. In the case of the waters, nitrate reduction was active to a depth of 50 meters. Morphologically, the organisms developing in these cultures were found to differ considerably with the different inocula. Some were plain rods, others resembled *Azotobacter* cells, while still others were undeniably spores.

TABLE X

Relative abundance of nitrate-reducing bacteria in the mud

Station number	Dilution of mud	Growth of bacteria	Microscopic examination	Gas evolution	Nitrite formation
1329	0.1	Turbid	Spores	++	++
1329	0.01	Turbid	Few cells	−	+++++
1329	0.001	0	0	0	0
1330	0.1	Turbid	Oval rods, spores (?)	−	+++
1330	0.01	Turbid	Oval rods, spores (?)	−	++++
1330	0.001	Turbid	Oval rods, spores (?)	−	−
1330	0.0001	−	−	−	−
1331	0.1	Turbid	Spores	−	+++++
1331	0.01	Some turbidity	Spores	−	+++
1331	0.001	Some turbidity	−	−	+++
1331	0.0001	−	−	−	−
1332	0.1	Turbid	Spores, rods	−	+++++
1332	0.01	Turbid	Spores, rods	−	+++++
1332	0.001	−	−	−	−

EXPLANATION OF NITRATE CYCLE IN THE SEA ON THE BASIS OF THE OCCURRENCE OF AMMONIA-OXIDIZING AND NITRATE-REDUCING BACTERIA

The results of the investigations carried out in connection with this expedition prove beyond any doubt that the marine bottom, both sand and mud, contains bacteria capable of oxidizing ammonia to nitrite, and later to nitrate; the process of nitrate formation in the sea must, therefore, be considered as bacteriological in nature. The results also show that the sea water and marine bottom contain bacteria capable of reducing nitrate to nitrite, in the presence of sufficient energy material, in the form of readily available carbon compounds. The fact that marine humus cannot be used as such a source of energy, while the amount of available carbohydrate in the mud is very limited, tends to emphasize that this process in the sea is only of questionable importance.

The problem finally remains to establish whether the limited occurrence of nitrite in sea water is due to the oxidation of the ammonia by nitrifying bacteria or to the reduction of nitrate by other bacteria. There is no doubt that the oxidation takes place chiefly at the bottom of the ocean; further that while the formation of nitrite from ammonium salts, under proper conditions of culture and with bottom material as an inoculum, can be readily demonstrated, nitrate formation takes place, under laboratory conditions at least, at a much later date. Reduction of the nitrate can be expected to take place both in the sea water and in the sea bottom; however, this process requires a supply of organic matter as an available source of energy. This can only be obtained through the photosynthetic activities of the planktonic organisms. It is no mere coincidence, therefore, that the maximum nitrite formation corresponds well with the maximum oxygen content in the water or the maximum photosynthetic activities.

The following hypothesis suggests itself at this particular point: Decomposition of the organic nitrogenous compounds takes place in the sea water but largely on the sea bottom, with the result that the ammonia is then liberated. This ammonia is rapidly oxidized by specific bacteria living in the bottom to nitrite and later to nitrate. This nitrate remains in the sea bottom and is not reduced, due to a lack of available energy for the nitrate-reducing bacteria and not to a lack of such bacteria. The small amounts of ammonia found in the sea water originate from the decomposition of the plant and animal residues in the plankton and in the water. The nitrate formed in the bottom gradually diffuses into the water, where it remains as such. On reaching the zone of photosynthetic activities, this nitrate is either consumed by the phytoplankton or is reduced by the nitrate-reducing bacteria to nitrite, which may also be gradually consumed by the plants. Very little nitrate reduction to gaseous nitrogen or complete denitrification is possible under normal sea conditions. Reduction of nitrate to nitrite does not mean necessarily any loss of nitrogen from the cycle of life in the sea.

Abundance of Marine Humus in the Bottom of the Gulf of Maine

A series of mud samples was taken with the small glass tube from the first four stations and from one sand station, in order to determine the abundance as well as the variability in distribution of the organic matter in the sea bottom. The methods of analysis, as well as the importance of these results in determining the origin and nature of the organic matter or the marine humus in the sea will be discussed in a

later publication. The organic matter is reported in terms of carbon and nitrogen. In order to calculate the organic matter content of the mud or sand from the carbon data, the factor 1.724, which has found extensive application in soil investigations, may be used, for reasons discussed elsewhere.

TABLE XI

Distribution of marine humus in the sea bottom, as shown by the carbon and nitrogen content. Percentage of dry material.

Station No.	Sample No.	Carbon content	Nitrogen content	C/N
1329	1	2.46	0.284	8.7
1329	2	2.50	0.290	8.6
1329	3	2.48	0.290	8.5
1329	4	2.46	0.282	8.7
1329	5	2.37	0.278	8.6
1329	Average	2.45	0.285	8.6
1330	1	2.65	0.304	8.7
1330	2	2.66	0.284	9.4
1330	3	2.62	0.306	8.6
1330	4	2.70	0.294	9.2
1330	5	2.81	0.288	9.7
1330	Average	2.69	0.295	9.1
1331	1	1.58	0.156	10.1
1331	2	1.54	0.148	10.4
1331	3	1.55	0.144	10.8
1331	4	1.36	0.133	10.2
1331	5	1.29	0.133	9.7
1331	Average	1.46	0.143	10.2
1332	1	0.82	0.072	11.4
1332	2	1.20	0.096	12.5
1332	Average	1.01	0.084	12.0
1335	1	0.084	0.008	10.5
1336	1	0.12	0.009	13.3

The results reported in Table XI show that while the variation in the organic matter content is very small in some of the stations, it is quite appreciable in others. These results point definitely to the conclusion that determinations based upon a single sample may frequently give quite inaccurate results.

The distribution of marine humus in the mud profiles taken from the various stations is given in Table XII. A heavy brass sounding tube was used for this purpose. The depth of the mud brought up by means of this corer ranged from 75 to 115 cm. As soon as brought up, the cores were divided into three or four sections and later each analyzed separately. It is important to remember, of course, that the mud was compressed by the sampler and that the actual depth of the mud was greater than that shown by the figures. The analyses of the total carbon and nitrogen were made on the materials which had been dried in an oven at 100° C. There has been some question, however, as to the influence of drying upon the changes in the nitrogen content of the mud.

TABLE XII

Relative distribution of marine humus with the depth of the mud profile. On basis of dry material.

Station No.	Depth of mud	Carbon content	Nitrogen content	C/N
	cm.	*per cent*	*per cent*	
1329	0–30	2.58	0.290	8.7
1329	30–60	2.45	0.250	9.8
1329	60–90	2.32	0.238	9.75
1330	0–30	2.74	0.296	9.25
1330	30–60	2.75	0.254	10.8
1330	60–90	2.60	0.236	11.0
1331	0–35	1.58	0.140	11.3
1331	35–65	1.64	0.130	12.6
1331	65–90	1.61	0.128	12.6
1331	90–115	1.57	0.126	12.4
1331	90–115 (reddish mud)	1.04	0.080	13.0
1332	0–25	0.67	0.050	13.4
1332	25–50	1.12	0.092	12.2
1332	50–75	1.43	0.096	14.9

The samples obtained from Station 1329 were, therefore, analyzed for total nitrogen both in a moist and in a dry condition and both sets of results calculated on a dry basis (Table XIII). These results show that while in some instances there is no appreciable difference in the nitrogen content of the mud as a result of its preliminary drying in the oven, in other cases the difference obtained may be appreciable. All the analyses reported here were made on the oven-dried (60–90° C.) mud, due primarily to the ease of handling and to the greater uniformity of the samples.

Stations 1329, 1330, and 1331 show a gradual even if only a very limited decrease of the humus content with an increase in depth of mud,

especially on the basis of the nitrogen figures. This is accompanied by a gradual decrease in the nitrogen content of the humus. This is due either to the greater decomposition of the nitrogen complexes with the age of the humus or to a difference in the nature of the materials deposited at different times. One is struck, however, by the great relative uniformity of the organic matter in the mud throughout the whole profile. One exception to this is found in one of the samples from the 90–115 cm. layer of Station 1331. This section of the profile was distinctly different in color from the rest of the profile and even from the duplicate part of another profile, being much redder in color. This proves again that even at one and the same station the mud may not be very uniform; this was brought out in Table XI for the data obtained from this station.

In the case of Station 1332, there is an increase in the humus content with an increase in the depth of the profile, as shown by both the carbon and nitrogen figures. This is due entirely to a difference in the

TABLE XIII

Influence of drying of marine mud upon its nitrogen content. On basis of dry mud.

Depth	Moist mud analyzed	Dry mud analyzed
cm.	*per cent N*	*per cent N*
0–30	0.306	0.290
30–60	0.282	0.250
60–90	0.239	0.238

nature of the material deposited at different periods, the surface material being much coarser than the lower layers. The amount of humus in the sand bottoms is very limited, usually equivalent to about 0.1 per cent carbon on the basis of the dry material. In view of the fact that no apparatus has been devised as yet to enable one to take a deep core of the sand bottoms, it is impossible to state how this humus content changes with the depth of the bottom. No information is available concerning any differences that may exist in the chemical nature of the humus in the mud and sand bottoms.

On comparing the data of the humus content in the mud (Table XI) with those of the bacterial numbers (Table IV), one finds a certain definite correlation. This is especially true when one compares Stations 1329 and 1330 with a high humus content, on the one hand, with Stations 1331 and 1332 with a low humus content, on the other. One must keep in mind, of course, the fact that the numbers of bacteria were determined

either in the very surface layer of the mud or in the 10 cm. layer, while the organic matter content was determined in the total core which ranged in depth from 15 to 25 cm. The bacteria are present most abundantly in the uppermost surface layer, where the organic matter is of recent origin and no doubt different chemically from the organic matter in the lower depths of the mud. The surface layer of the mud can be looked upon as the layer most active bacteriologically. The same is probably true of the sand, where the relative abundance of bacteria is considerably greater than the relative humus content. The abundance of humus can, therefore, be looked upon as only one factor controlling bacterial numbers and activities.

Summary

A bacteriological survey has been made of the waters and bottom sediments in the Gulf of Maine and Georges Bank. The samples of water, plankton tow, and sedimentary material were taken under sterile conditions and subjected immediately to bacteriological analysis as soon as brought on board the " Atlantis."

The agar-plate method was used for the enumeration of the numbers of bacteria. This was supplemented to a limited extent by the dilution method. Various specific media were used to determine the distribution and relative abundance of certain groups of bacteria which are believed to take part in important marine processes.

The results obtained demonstrated the fact that the bacterial population of the sea can be divided into three groups on the basis of their habitat: (1) those forms which live in the sea bottom, especially in the surface layers; (2) those bacteria which live in the free water, this being possible only when the water contains in solution organic and inorganic substances which can serve as nutrients for the bacteria; (3) those bacteria which live largely upon or in association with the plankton organisms.

Sea water is a rather poor medium for the growth of bacteria, while the marine bottom is comparatively richer in the total number of bacteria capable of developing on the plate and in solution media. Mud bottoms contain more living bacterial cells than sand bottoms. However, the waters above the sandy bottom were found to contain many more bacteria than the waters above the mud bottoms. This may be due to the greater abundance of plankton organisms, especially diatoms, in the shallower seas with the sandy bottom, to the greater mixing of the waters, or to the greater absorption of bacterial cells by the mud bottom material than by the sand bottom.

The numbers of bacteria obtained by the plate method represent only a part of the bacterial population of the sea. This was shown by the fact that the dilution method, using gelatin as a medium for bacterial development, gave higher numbers than the plate method.

A decided parallelism was observed between the abundance of diatoms in the sea and abundance of bacteria. A comparatively large number of these bacteria were agar-liquefying organisms. In the artificial culture of diatoms, the numbers of bacteria were found to increase with the development of the diatoms. However, when the latter reached a maximum and began to die out, bacterial numbers did not diminish very rapidly; these bacteria seemed to be largely limited to a few specific types, the importance of which in marine processes still remains to be determined. These results seem to point definitely to the fact that the development of phytoplankton in the sea is accompanied closely by bacterial development. The bacteria feed upon the excretion products of the diatoms, algæ, and animal forms and probably upon these plankton forms themselves as soon as they die, thus bringing about their rapid disintegration and liberation of the nutrient elements in an available form.

Anærobic bacteria were found abundantly in the marine mud; these bacteria were present in the mud even at considerable depths. Their presence points to continued decomposition of the plant and animal debris of the ocean on and in the ocean bottom, even with an insufficient supply of oxygen.

The bacterial population of the sea was found to consist of a number of types, some of which take part in well-known processes which are of great importance in the metabolism of the sea, such as nitrogen-fixing, nitrite- and nitrate-forming, nitrate-reducing, cellulose-decomposing, agar-decomposing, chitin-decomposing, and many others. The importance of many common bacteria, both ærobic and anærobic, mostly non-spore-forming but also spore-forming, in marine processes still remains to be determined. The presence of nitrogen-fixing bacteria, comprising both the ærobic *Azotobacter* and the anærobic *Clostridium*, has been definitely demonstrated. One strain of the first group was isolated in pure culture and was found to be *Azotobacter chroococcum*, which fixed considerable quantities of nitrogen when grown on artificial culture media, with various carbon sources.

Bacteria capable of oxidizing ammonium salts to nitrites were found in the sea bottom, but only seldom in the sea water. For demonstrating the presence of these organisms in the sea, a medium containing a layer of sand and $CaCO_3$ and covered with a shallow layer of liquid containing an ammonium salt and the necessary minerals was used.

Bacteria capable of reducing nitrate were found abundantly both in the water, especially at the surface layers and the zone of photosynthetic activities, and in the sea bottom. However, these bacteria were able to reduce nitrate only to nitrite and not to nitrogen gas; only in one instance, namely, in the mud from the first station taken on this expedition, was there present an organism capable of bringing about the last process.

The cycle of nitrate in the sea is explained as follows: The nitrogenous constituents of the plant and animal residues in the sea are decomposed with the liberation of ammonia, largely in the sea bottom. The ammonia is oxidized in the sea bottom to nitrite and later to nitrate. The latter gradually diffuses into the waters. It is not reduced in the sea bottom or lower layers of water, largely because of a lack of available energy material necessary for the activities of the nitrate-reducing bacteria. In the zone of maximum photosynthetic activity such energy is available, hence nitrate-reduction may take place, but only to nitrite, since bacteria capable of reducing it to nitrogen gas are lacking under those conditions. The nitrite thus formed may also be assimilated by the phytoplankton and does not represent any loss of nitrogen in the sea.

The marine humus (total organic matter) content of the mud bottom in the Gulf of Maine is found to be more or less uniform in composition. This humus is best calculated from the total organic carbon. It was found that determinations based upon one sample may not give absolute evidence, since different samples may give considerable variation. The humus content usually decreases with the depth of the mud. In some stations, however, an increase in humus content with depth was observed. There is also a widening of the carbon-nitrogen ratio of the humus with an increase in depth. This points to a greater decomposition of the humus, especially of the nitrogenous complexes, with an increase in depth. An increase in humus content with depth of mud found in some stations merely points to the fact that the rate of deposition of the humus may vary considerably with different periods.

A definite parallelism was observed between the numbers of bacteria and abundance of humus in the mud.

A detailed review of the literature bearing upon the results presented in this report, as well as a detailed description of the methods used and a correlation of these results with the results of bacteriological investigations of the sea near shore, is reserved for a future publication.

THE OVARIAN CYCLE IN THE VIVIPAROUS TELEOST XIPHOPHORUS HELLERI

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A number of teleostean families include viviparous genera. These vary in type of reproductive adaptation from ovoviviparity, where the ova are merely retained in the ovary until hatching, to true viviparity where elaborate nutritive organs are formed by both parent and offspring. The Pœciliidæ, to which *Xiphophorus* belongs, includes oviparous, ovoviviparous, and truly viviparous forms, many of which are commonly raised in aquaria.

The first description of the development of a pœcilid is by Duvernoy (1844) on *Pœcilia surinamensis.* Wyman (1856) described the development of the " four-eyed fish," *Anableps gronovii,* which he found to be truly viviparous, the yolk-sac serving as an absorptive organ. Ryder (1882) described the development of *Gambusia* and its ovarian gestation. He again published (1886) his material as part of a longer paper on the development of viviparous osseous fishes in which he reviews all the early work. To this day his paper is the best single work treating with this phase of the subject. He found *Gambusia* to be ovoviviparous and described a number of embryological stages.

Zolotnitsky (1901), recording the habits of *Girardius decemmaculatus,* describes the mating and parturition, ascribing to the male the function of midwife; the male no doubt does show interest in the act but probably not from that motive. Zolotnitsky includes a series of observations on length of reproductive cycle and temperature. Philippi (1908) described in detail the gross and microscopic structure of both male and female reproductive tracts of two species of *Glaridichthys.* This paper is the most detailed one for any member of this family. As to the female reproductive system, he discusses the method of entrance of sperm into the follicle and of sperm storage, as well as the normal histology. Kuntz (1913), discussing the habits and the morphology of the reproductive organs of *Gambusia,* supplements and in a few places corrects Ryder's work. In general the embryology is shown to be almost identical with that of oviparous forms. Hildebrand (1917) carried on much the same sort of research, adding somewhat to the contributions of Ryder and of Kuntz. Milewski (1920) repeated much of

Philippi's work, and included also a number of other genera. He treats of the structure of the female and male organs (of which he distinguishes several types), mating, fertilization, parturition, sexual dimorphism, hybrids, etc.

These previous works are all from the point of view of the embryology and the anatomy of the reproductive tract. The subject may, however, be approached from the point of view of the ovarian cycle with its adaptations in their time relation to the cycle as a whole. The discussion resolves itself into two chief parts; first, a consideration of means of dating the pregnancies, and second, a description of the changes in the ovary and its contents during the cycle.

Material

Xiphophorus was selected because of its convenient size, hardiness in captivity, and apparently delicately poised sexual balance, as indicated by normal sex-reversals (Essenberg, 1923, 1926). The individuals used were mostly young breeding fish about 50 mm. in length and weighing about 1.5 grams. This size was preferred as the ovary is small enough for ready fixation while containing enough ova to allow material for weight and embryological observations to be taken without spoiling all of it for histological use. The offspring of large and small individuals do not differ noticeably in size, but the large females have larger litters than do the small ones.

Female Genital Tract

The ovary of *Xiphophorus* is a large unpaired body which in mature fish occupies most of the posterior part of the peritoneal cavity. Enveloping it is a thin, slightly pigmented peritoneal membrane. When this is removed the follicles or ovisacs appear lying quite separately, like grapes in a bunch, making up the bulk of the cortex. The follicle is composed of a single layer of cells supported by a thin theca, through which the contents are readily visible. Between the large follicles, with their golden amber ova, can be seen numbers of small white ova, usually located close to the central cavity and chiefly along its dorsal wall. These eggs form a continuous series from about 12 micra to 0.5 mm. in diameter. In shape they are commonly spheroidal but are often irregular, especially in preserved material. Close to the ovarian lumen is the conspicuous ovarian vein with small veins leading into it from a circular sinus on each follicle. These venous circles—described for *Gambusia* (Ryder, 1885)—are most distinct in the gravid ovary, becoming much less noticeable in the later stages of pregnancy. Penetrating to each ovum is a funnel-like invagination of the ovarian lining which

widens out against the follicular epithelium at the bottom. This is called by Stuhlmann and by Philippi a *delle*. The sperm has access to the mature ovum at fertilization through a minute pore in the epithelium of the bottom of the delle. Later this opening enlarges to form the birth-pore through which the young escape into the central cavity at parturition.

Posteriorly, the ovarian cavity is continuous with that of the oviduct. This is not a homologue of the Müllerian duct, but is a single median tube arising from two primordia (Essenberg, 1923). One primordium is the posterior part of the ovary; the other the lining of the body cavity near the urogenital sinus. This oviduct is a short muscular tube elaborated into deep longitudinal folds, in the epithelium of which the sperm is stored, the sperm heads being visible within the high columnar cells. As the oviduct passes ventrally it widens and becomes less folded, then contracts sharply to an isthmus where the walls thicken to form a strong sphincter. Beyond this valve the oviduct again widens and runs to the tip of the genital papilla lying in the urogenital sinus which it enters from the anterior margin and very largely fills.

Procedure

A graded series of pregnant females was required from which other material could by comparison be assigned to definite stages. Data were taken on a random representative sample of fifty pregnant fish as to total length, length to end of scales on tail, greatest depth, depth of caudal peduncle, and body weight. The ovaries were opened and usually six ova or embryos removed. After measuring two or three representative embryos, three were dried, the others fixed in Bouin's with the remainder of the ovary.

The series was then arranged in order from the earliest to the latest stages of gestation. For the early stages, particularly those of growth of ova, the diameter of the egg was used as a criterion of developmental advancement; later the degree of differentiation of the embryo; finally the length of the embryo and size of the yolk sac. Now assuming the sample to be representative, the youngest stage should be post-parturient, which comparison with ovaries of known post-parturients showed to be true. Similarly the oldest stage should be pre-parturient—its ovary contained embryos indistinguishable from new-born fish—and the median fish should be halfway between, or should have passed through 50 per cent of the cycle. In this manner the fish were assigned percentage positions, so that the fifth fish was 10 per cent, the tenth 20 per cent, etc. This method of marking stages was deemed best because it represents a variable time interval which changes with the length of the gestational

period. As under greenhouse conditions this period is about thirty days, 1 per cent equals about eight hours. For convenience the stages are indicated by the numeral alone, as, position 50.

Some justification perhaps is necessary for basing this curve on a sample of only fifty individuals. Undoubtedly it is to some extent inaccurate, but it appears to be sufficiently exact for practical use. First, there are no large gaps nor overlaps in the seriation—diameters and lengths follow smooth curves and show gradual increases. Second, several observations on fish kept under constant temperature conditions indicate much the same time relations. Third, the embryos of a litter are not all identical in size or degree of development, so that an appreciable overlap often exists between two adjacent series of young. For example, in a litter of new-born fish length varied from 8.6 mm. to 9.3 mm. As can be seen, a curve might be made more accurate than would be significant. Parturition, the only end-point for experimental timing, lasts a number of hours and may even be protracted over a period of several days.

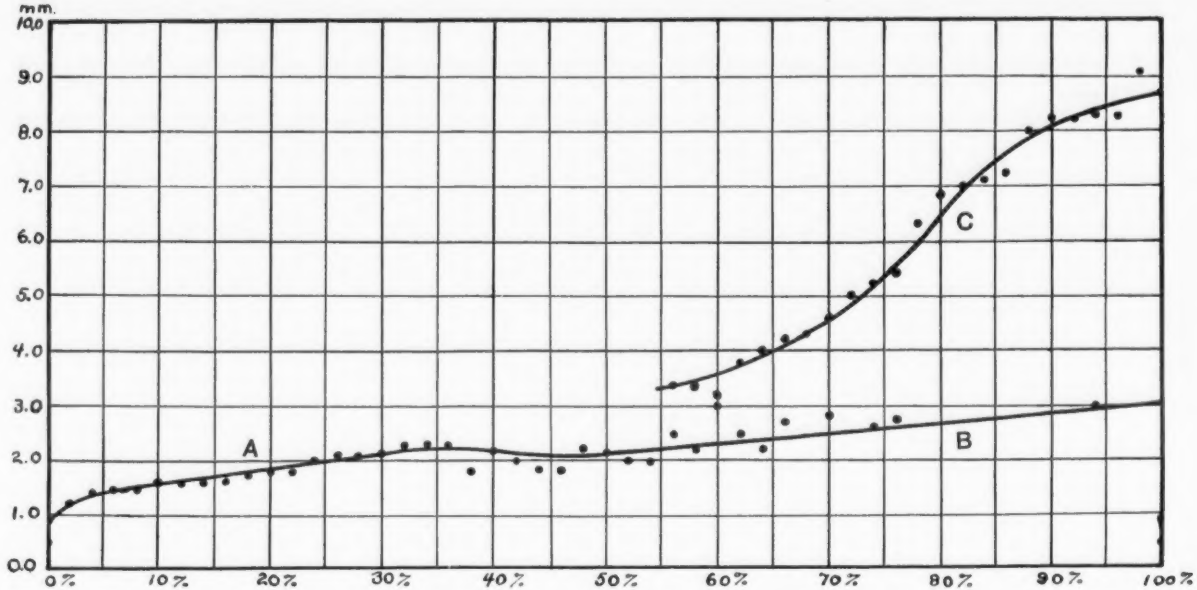
Means of Dating Pregnancies

Ovaries may be assigned to relative positions in the cycle by comparison with a dated series, or more conveniently with Text Fig. 1, on which position is plotted against diameter of ovum, diameter of ovisac, and length of embryo, the chief criteria used in arranging the dated series. Between 30 and 50 differentiation of the embryo is very rapid and affords one a useful means for determining chronological position.

The desirability of a means of dating pregnancies by external criteria led to a study of body form and proportion using the ratio of greatest depth to depth of caudal peduncle and the weight length relations by means of the standard constant of conditions formula,

$$K = \frac{W}{L^3}.$$

The changes in proportion due to the stage of pregnancy were obscured by irregular variations ascribable to amount of body fat and to intestinal content. Consideration was also given to the "pregnancy spot" and to the scale markings. All four external criteria proved unreliable means of dating pregnancies, so that in practice the fishes had to be killed before their position could be ascertained. However, the "pregnancy spot" was found reliable as an indication of sexual maturity. Reproduction appears to play a comparatively constant rôle in the general physiology of the female *Xiphophorus*.



TEXT FIG. 1. Growth of the ovum, embryo, and ovisac; *A*—diameter of the ovum, *B*—diameter of ovisac, *C*—length of embryo.

The Yolk and the Follicle

A normal ovary contains, besides the group of large eggs, numerous smaller ones, ranging from ova with a diameter of 10 to 12 micra to large ones of about 0.5 mm. in diameter. Essenberg (1923) describes the origin of the definitive ova from the epithelial lining of the ovary, confirming Philippi's observation on *Glaridichthys*.

The smallest ovum with a follicle illustrated by Essenberg is one of 12 micra in which four or five follicular nuclei are seen around the ovum. The germinal vesicle is large and central. Fig. 1, Plate I, although a slightly older ovum, shows the same picture. The cytoplasm is quite strongly basophilic and the germinal vesicle is achromatic, showing two very chromatic nucleoli. The follicle is of very low squamous epithelium. As growth continues the cytoplasm becomes less basophilic (Fig. 2, Plate I). Here the germinal vesicle is even less chromatic than earlier, the only strongly basophilic portion being the large nucleolus. Around the low follicular epithelium the stroma is condensing to form a theca. The water content now must be very high as ova are often badly shrunken or collapsed if treatment is at all drastic. This is about as large as ova with homogeneous cytoplasm are found, for soon oil droplets appear in the borders of the cytoplasm. With the commencement of oil secretion, the epithelium begins to thicken, becoming cuboidal (Figs. 3 and 4, Plate I) and eventually moderately low columnar in type. Figure 4 shows a change in follicular epithelium with a rapid transition from cuboidal to columnar. The epithelium, which is high for follicles of this size, shows undulations as if to increase the surface; this is quite common. Philippi illustrates for *Glaridichthys* a series of follicular trabeculæ penetrating the yolk. My material does not show this except where it is an obvious artifact due to shrinkage or rupture of the ovum.

When a diameter of about 450 micra has been reached yolk secretion begins, a number of minute yolk granules appearing along one side of the ovum near the periphery. Those granules nearest the follicular epithelium are the most minute; those found more centrally are larger. Figures 5 and 6, Plate I, are of opposite sides of one ovum, and show a marked difference in the character of the epithelium and of the cytoplasmic inclusions. One side, Fig. 5, still contains only oil; the other, Fig. 6, both oil and yolk. As the yolk secretion continues, the entire mass of the ovum becomes filled with globules of oil and yolk. In histological preparations the oil is dissolved out, leaving cavities in the cytoplasm. The germinal vesicle still is achromatic and central in position. Such ova reach 0.5 mm. in diameter about parturition or immediately thereafter.

At this time these ova, which will form the next litter, are growing rapidly toward maturity. The follicle cells are cuboidal and very active. The ova are divided into two portions: centrally a structureless mass of coalesced yolk, peripheral to which is a layer of cytoplasm full of oil globules and yolk (Fig. 7, Plate I). This central yolk mass increases at the expense of the peripheral layer. The follicular epithelium is gradually reduced in height, probably due to two factors: (1) its physiological secretory function, and (2) the mechanical necessity for the single layer of cells to continue to cover the ever increasing surface of the ovum. At 30, after fertilization, the ovum and the follicle separate so that the ovum floats free in the cavity of the follicle which, from this point, is often called the ovisac (Fig. 8, Plate I). The ovum now is a mass of yolk with oil droplets embedded in its periphery. On one side is the blastodisc from which a thin membrane runs out onto the yolk. None of the material of this age shows a distinct vitelline membrane although one is found later surrounding the embryos. The follicle again consists of squamous epithelium supported by a vascular theca. As from this stage on the embryos and the follicle are not in contact, they are perhaps best discussed separately.

Throughout the period of gestation the epithelium of the follicle appears inactive, and becomes progressively thinner as does the theca, reaching an extreme of tenuity just before parturition (Fig. 9, Plate I, and Fig. 1, Plate II). Coincident with the development of the embryo there is an increase in the vascularity of the follicle.

At parturition the ovisac commences a new phase of activity (Text Fig. 3, *B*). As it collapses the squamous epithelium rapidly hypertrophies. The cells are columnar and are attached to each other only at the base (Fig. 2, Plate II). This strange epithelium has a windswept,

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Explanation of Plate I

Camera lucida drawings from Delafield's hæmatoxylin and eosin preparations. × 453. *Y*—yolk, *T*—theca, *O*—oil, *F*—follicular epithelium, *P*—peritoneum, *S*—secretion.

FIG. 1. An early ovum showing germinal vesicle nucleoli, and follicle.

FIG. 2. An older ovum showing follicle with a theca.

FIG. 3. An ovum, average diameter 200 micra, showing beginning of oil secretion.

FIG. 4. An ovum, average diameter 310 micra, showing transition from cuboidal to columnar epithelium.

FIG. 5. An ovum, average diameter 450 micra, showing late stage of oil secretion.

FIG. 6. The opposite side of the same ovum, showing the commencement of yolk secretion.

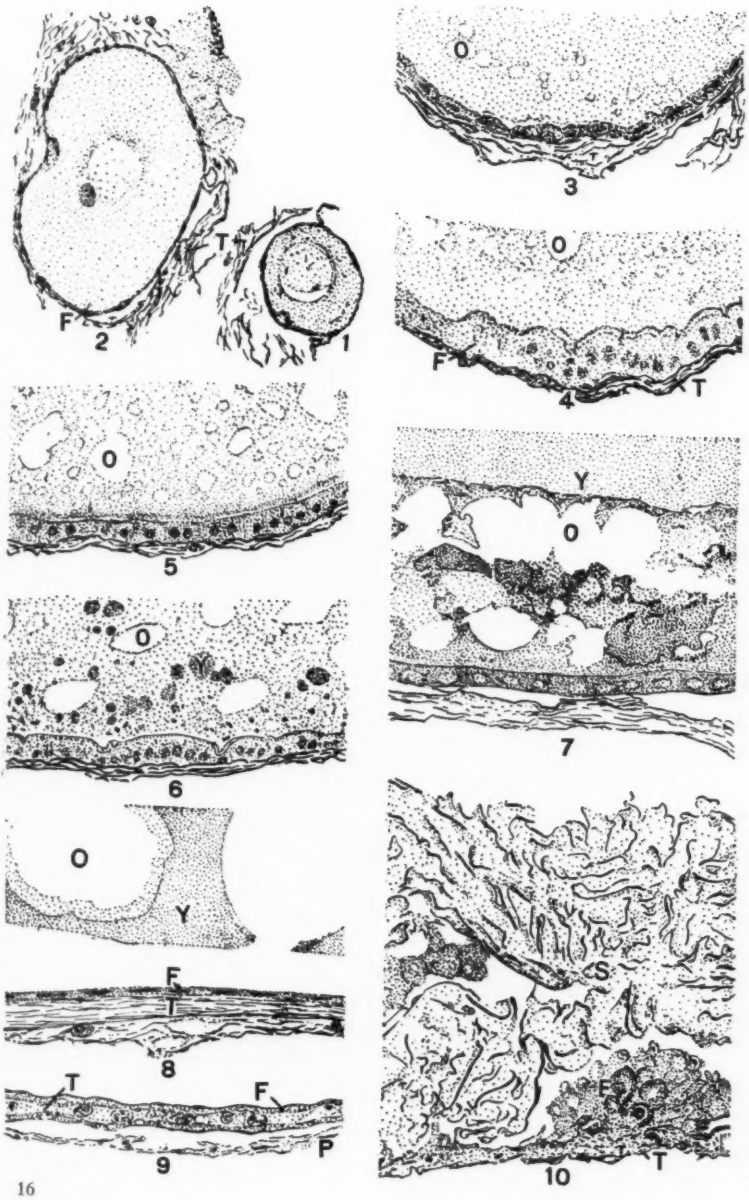
FIG. 7. An ovum at 10, showing yolk secretion at its height.

FIG. 8. An ovum at fertilization—the cessation of yolk secretion.

FIG. 9. Wall of an ovisac at 100, showing its vascularity.

FIG. 10. Wall of a spent follicle, showing it full of secretory material.

PLATE I



fringed appearance. The nuclei are achromatic and are found well up from the base as if to indicate considerable activity. Beneath the follicular epithelium, the theca has also greatly thickened and bears numbers of polygonal cells. The blood vessels also are enlarged and form a zone in the thickness of the follicle wall (Fig. 3, Plate II). The follicle then begins to secrete, Fig. 4, Plate II, and to slough, so that its lumen is filled with the secretory product, which resembles a mass of crumpled membranes with here and there some cell fragments. This secretion passes into the lumen of the ovary (Fig. 10, Plate I, and Fig. 5, Plate II).

Vitelline Plug

Within the vitelline membrane of ova at about position 70, a plug is to be found closing the birth-pore. This is composed of a mass of vesicular material resembling in structure the cytoplasm of ova prior to the commencement of yolk secretion (Text Fig. 3, *A*). This mass is found just opposite the birth-pore as if it had been first loose and then settled in the slight bulge where the vitelline membrane bellied out into the lumen of the ovary. As to its nature, it may be any of three things, i.e., nutrient material from the parent, excretory product of the embryo, or a secreted reinforcement for the membrane across the birth-pore. This last seems the least probable suggestion, as this membrane in *Xiphophorus* is really only a functionless rudiment. The plug seems rather too firm and solid to be an excretory product; moreover, the embryo has no fecal material in the gut and since no goblet cells are present as yet, the plug is not apt to be mucous in nature. It seems most likely to be either a nutritious substance newly secreted from the follicles, as in *Anableps,* or modified yolk cut off from the yolk mass prior to its complete enclosure by the embryo.

Structural Changes in the Embryos

The embryology, as before mentioned, has been described in this family. The various authors agree that the development of viviparous forms differs in no significant particulars from that of related ovi-

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Explanation of Plate II

Figures 1 to 4, × 320. Fig. 5, × 72.

Fig. 1. A section showing relation of embryo and follicle just prior to parturition. *Y*—yolk in yolk-sac of embryo, *Ys*—yolk-sac, *V*—vitelline membrane, *F*—follicle.

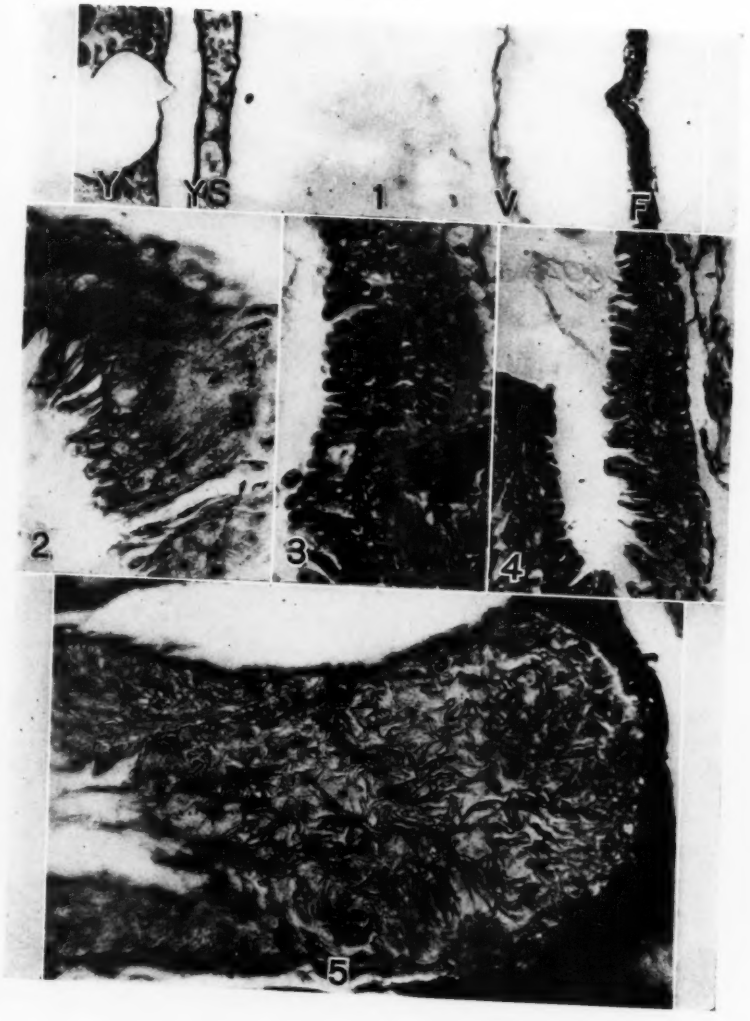
Fig. 2. Wall of collapsed follicle showing hypertrophied theca and the follicular cells.

Fig. 3. A slightly later stage showing the zone of capillaries.

Fig. 4. The follicle just beginning to secrete.

Fig. 5. A follicle at the height of secretion.

PLATE II



parous species. This section does not purport in any way to describe the embryology or to give a complete picture of any stage or any system, but to present such conspicuous characters as would be of practical use in determining the age of the material, especially in sections of the ovary where the embryos may be cut through any plane. Fertilization stages have not been found but they occur at about 30. At this time the ovum has reached a diameter of 2.1 mm. and consists, except for the blastodisc, of a solid ball of yolk with numerous large oil drops around the periphery. Fertilization does not seem to take place simultaneously in all of a batch of ova, so that different stages of cleavage can be observed in the same ovary. Figure 3, Plate III, shows one of the blastodiscs from an ovum at 30, which is composed of about 60 cells.

At 40 (Fig. 4, Plate III) the blastodisc has spread and formed the germ ring on one side of which the embryonic shield has just appeared. The shield lengthens and development is rapid so that at position 50 the embryo is well formed.

The embryo (Fig. 5, Plate III) is about 2 mm. long and has already produced an excellent vitelline circulation. Well-formed optic cups with lenses in them are visible externally. The cup is differentiated into retina and choroid coats and the choroid fissure still remains. Somewhat posteriorly the large spheroidal otic vesicles appear. The lateral ventricles of the brain are beginning to grow and the myelencephalon shows its characteristic thin dorsal wall, although the nervous tissue is not as yet differentiated into white and gray substance. One also may note that the anterior neuropore is open, the tail is free on the yolk, and that there are still no limb buds. The notochord is differentiated in the posterior part.

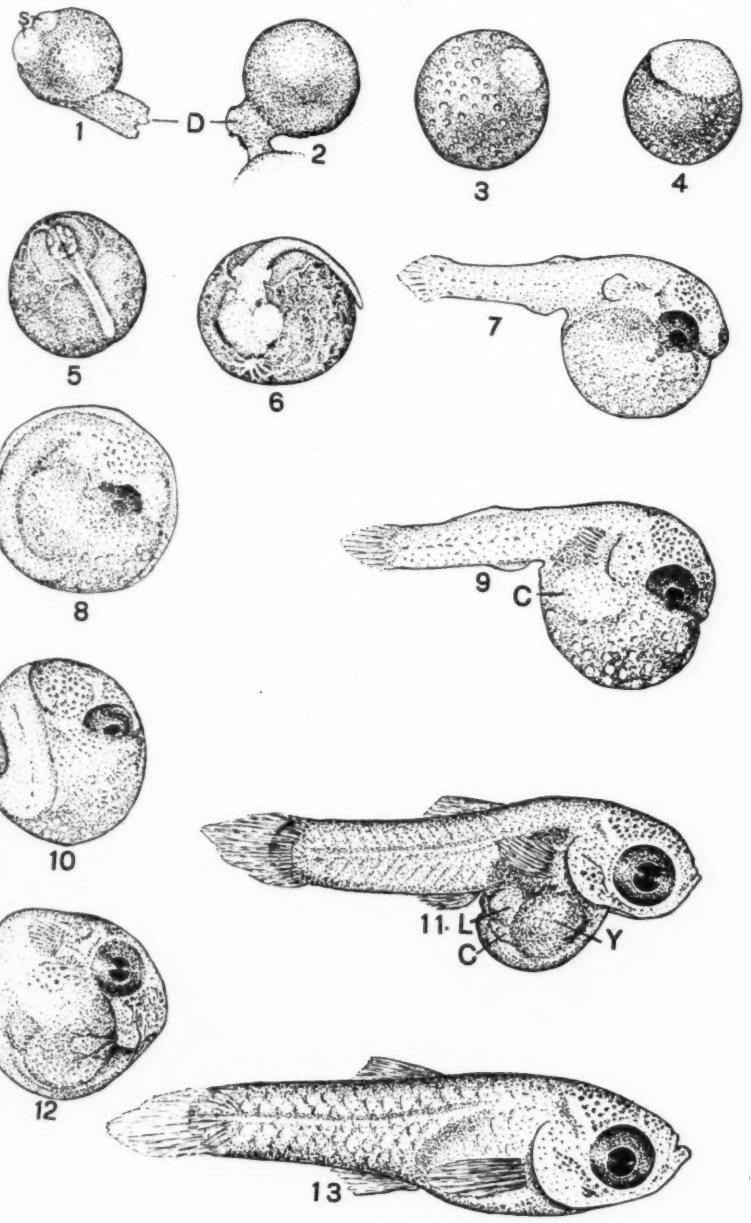
At 60 the embryo has reached a length of about 3.6 mm. (Fig. 6, Plate III). The embryo has proportionately a very large head to which

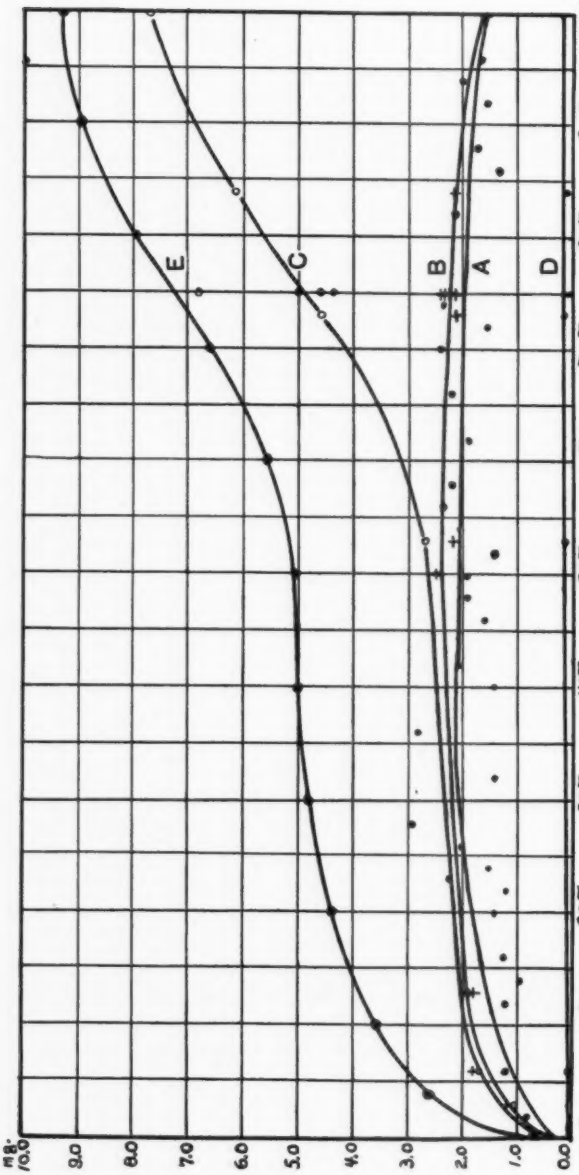
Explanation of Plate III

Camera lucida drawing × 11.25. Embryos fixed in Bouin's and cleared in alcohol and glycerine.

FIG. 1. Ovum in follicle at 10. *S*—small ovum in theca of large one, *D*—attachment to center of ovary; this stalk contains a delle.
FIG. 2. Ovum in follicle at 20.
FIG. 3. Ovum at 30 showing a late cleavage stage.
FIG. 4. Ovum at 40 showing an early embryonic shield.
FIG. 5. Embryo at 50.
FIG. 6. Embryo at 60, showing pigmented eyes and limb buds.
FIG. 7. Embryo at 70.
FIG. 8. Embryo at 70 enclosed in the ovisac.
FIG. 9. Embryo at 80, *C*—cœlom.
FIG. 10. Embryo at 80 in its customary position.
FIG. 11. Embryo at 90, *Y*—yolk, *C*—cœlom, *L*—liver.
FIG. 12. Embryo at 90, coiled in the ovisac.
FIG. 13. Young at birth.

PLATE III





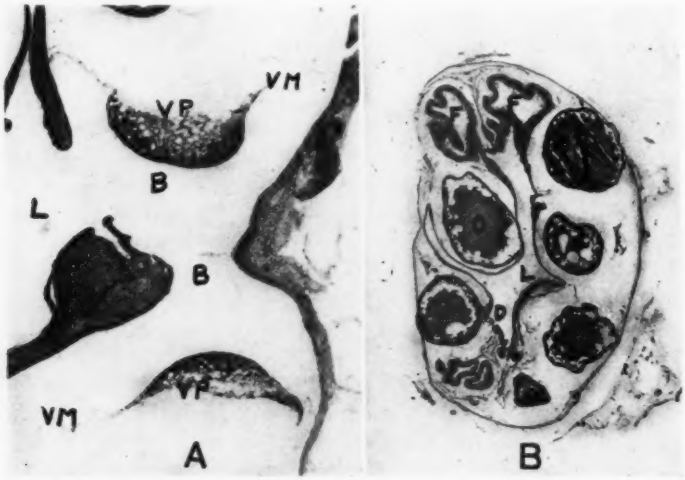
TEXT FIG. 2. Composition of ova and embryos; *A*—dry weight from dated series, *B*—organic content, *C*—water, *D*—ash, *E*—total weight, i.e., total of *B*, *C*, and *D*.

the trunk, with its newly acquired limb buds, appears as an appendage. The eye shows considerable advance, pigmentation being in progress. The lens is fibrous and the retina is separating medially into the ganglionic and nuclear layers. Elsewhere the nervous tissue is differentiated into gray and white substance, and a number of nerves have appeared. The beginning of histogenesis of cartilage and muscle occurs at about this time. Development of the digestive tract has resulted in a flattened pharynx lined with cubical epithelium and a tubular gut lined with columnar epithelium. Filling a considerable part of the cœlom is the liver anlage, a highly vascularized mass of rather darkly-staining polygonal cells. Another new organ is the pronephros, which here is only a slightly bent tubule just posterior to the pharynx.

Figures 7 and 8, Plate III, show the general appearance of an embryo at 70 in the ovisac, and released. At this stage, 4.6 mm., the head, although large, does not overwhelm the body but seems to fit it. The snout tends to be buried in the yolk up to the eyes or above. Borne upon the trunk, which usually is curled slightly to the side, are dorsal, ventral, and caudal fins. Only in the caudal fin are any fin rays to be found although the cartilages of the pectorals are formed. Pigmentation has now progressed so that chromatophores are abundant over the cerebrum and are occasionally to be seen on the caudal peduncle. Internally the peritoneum is also pigmented. Sections show the olfactory anlage to be still only thickened placodes; while the otic vesicles have developed and are now no longer round but irregular, due to the marking out of the semicircular canals. A pair of thin cartilaginous walls rises one on either side from the otic capsules to protect the brain. The spinal cord is protected similarly as there are cartilaginous lamellæ in the neural arches of the trunk and in the hæmal arches of the tail; these latter, however, are very minute. In the pharyngeal region the thyroid anlage forms a pit in the floor of the pharynx. On either side are the visceral pouches, which so far contain no gills. Curled under these and mostly out of sight are the operculæ. The pineal body and the pituitary are now well developed.

Figure 9, Plate III, shows an embryo at 80 free, and Fig. 10, a littermate in the position customarily taken in the ovisac, with the tail flexed and held over the back of the head. The snout is slightly embedded in the yolk. The pigment cells are distinct along the lateral line of the tail. Within the posterior part of the yolk sac is a clear vesicle, the enlarged cœlom. Dermal fin rays have now appeared in the pectoral fins. In the region of the otic vesicles the cranium is beginning to roof over the brain. The ribs are now present. Pharyngeal teeth are found although so far but little dentine has been deposited. The olfactory

apparatus is a deep flask-shaped pit with a thickened sensory epithelium at the base. Thickened sensory areas are also to be found in the lining of the canals and ampullæ of the ear. A cross-section of the pharynx shows distinct gill bars which, with the exception of the last, bear no filaments. Medial to the gill bars along the ventral aorta the thyroid appears, consisting of scattered follicles full of colloid. The viscera have changed somewhat; the gall bladder which earlier was lined with cuboidal cells now has a squamous lining; distinct pancreatic rudiments are present in the mesenteries. The cells of the gas gland have become large and achromatic.



TEXT FIG. 3. *A*. Section showing vitelline plugs closing the birth-pores. × 72. *L*—lumen, *Vp*—vitelline plug, *Vm*—vitelline membrane, *B*—birth-pore. *B*. Section of an ovary at 10 × .15. *O*—ovum, *F*—empty follicle, *D*—delle.

At 90 the *Xiphophorus* embryo is so long that in the ovisac the animal forms a complete loop, keeping its tail on the anterior part of the head. When released it straightens out to about 8.1 mm. (Figs. 11 and 12, Plate III). The yolk sac is an irregular mass which in preserved material shows a number of distinct parts. Anteriorly are the heart and vitelline veins, then the yolk proper which as an almost bipartite mass occupies ventral and lateral positions. In the notch between the halves of the yolk are two swellings, the liver and the cœlom. The operculæ now are conspicuous as they stand out from the head and are studded with many large chromatophores. The dorsal and anal fins

have fin rays as well as the pectorals and caudals. The pelvic fins are represented only by thickenings just anterior to the anus. A section through the optic chiasma shows large lymph sinuses in the orbit and between the eyes. Below in the lower jaw is a tongue cartilage which forms a distinct inverted U. A sheath is now being laid down upon the notochord. In the body cavity changes are slight; the kidneys are much more vascular than before and show the capsules around the glomeruli. The lining of the stomach and intestine is thrown into folds sparingly supplied with goblet cells.

At parturition (Fig. 13, Plate III), we find a perfectly formed little fish about 8.9 mm. long, short and plump, with a smoothly rounded belly, quite capable of looking after himself and ready to feed or to dodge his cannibalistic relatives. Recently he has acquired a set of scales and greatly elongated his pectoral fins. The retraction of the yolk seems to be accomplished chiefly by the removal of cœlomic fluid, the place of which is taken by the yolk and liver which, up to this point, have projected far below the normal body contour. The yolk now surrounds the truncus arteriosus on three sides, lying dorsal to the heart and between the ducts of Cuvier, extending throughout and filling almost the entire body cavity. The stomach, intestines, and skin are copiously supplied with goblet cells.

Weight and Composition of Ova and Embryos

In the course of development, changes take place not only in form and differentiation but also in weight and composition of the ova and embryos. Text Fig. 2 shows the results of two sets of weight measurements. *A* represents data derived from the dried embryos from the dated series. These were air-dried upon cover-slips, weighed on the slip, then removed by scraping and the weight of the cover-slip determined. Weights so obtained were relative but not exact as the degree of desiccation and the completeness of removal from the slip were variable. The slight residue left on the slide makes this curve uniformly low. Curve *B* presents data derived from a special series where quantitative determinations of organic content were made; curve *C* for measurements of water content; curve *D* for ash; and curve *E* for total weight. The number of individuals used for the determinations was governed by the number which could be dissected intact from the ovary, i.e., eight to twenty-five.

The most interesting of these curves have to do with the dry weight of the embryo and its parallel, the organic content of the embryo. These two curves are so similar that they may be discussed as one. The ova are shown to increase in weight until maturity, then upon fertilization

at 30 the increase gradually ceases and a slight decline begins. This is ascribable to the utilization of the yolk in the normal metabolism of the embryo. The two curves differ slightly in that *A* is fitted most perfectly by an almost straight line from 30 to 100 while from 70 to 100 *B* fits a more strongly curved one, indicating a slight increase in rate of yolk consumption. *B* appears more reliable than *A* as the weights were determined with greater accuracy. If *D*, the ash content, were added to *B*, it would then be exactly comparable to *A* and would more nearly show the same straight line picture.

The data on ash content are not of much significance, being almost within the error of the determination. At a maximum it is but a little over 0.1 mg. per embryo. It does, however, suggest that, unlike organic content, mineral salts continue to increase in quantity after fertilization, being taken up as needed by the embryo.

As the yolk decreases and the ash content is small, the obvious increase in volume of the embryo is seen to be due to absorption of water. During the early stages of egg development, the percentage of water is high as small ova shrink easily and are distorted in many sections. As the deposition of oil and yolk commences, the organic material accumulates faster than does the water so that water content falls nearly to 50 per cent, which it approximates until 60. When yolk ceases to be laid down, the percentage of water is increased by continued absorption. This absorption continues at a rate of increase corresponding to the growth of the embryo, the water content reaching at birth about 84 per cent of the total weight. Total weight represented upon Text Fig. 2 by Curve *E*, which is the summation of *B*, *C*, and *D*, shows rapid increase until fertilization—the period of yolk secretion—then, a pause followed by a notable increase—the period of water absorption.

Discussion

While ordinarily pregnancies are readily classified as either viviparous or ovoviviparous, it is not always easy in practice to determine the nutritive status of the embryos without obtaining their dry weights at various stages.

In several families, Embiotocidæ and Zoarcidæ, the young feed upon a cellular secretion or ovarian milk but the presence of material within the follicles or the lumen does not seem entirely diagnostic of true viviparity. *Xiphophorus*, a distinctly ovoviviparous species, has material within the vitelline membrane—the vitelline plug. As the exact nature of this material is unknown, little can be said other than that suspicion might be thrown upon a claim concerning the nutritive status of a teleost embryo, where dependence was placed upon this criterion alone.

Absence of special organs of absorption in the embryo does not necessarily indicate the ovoviviparous condition; any body surface, if somewhat permeable, may function as a pseudo-placenta. In the pœcilids the condition of *Anableps*, in which the young are retained and nourished in the ovarian lumen, can be derived from that of *Xiphophorus* by two relatively minor changes: (1) a continued secretion by the follicular cells after their separation from the ovum, and (2) some membrane of the embryo remaining permeable to certain large molecules such as those of fatty acids or amino-acids.

The anatomy of the ovary of *Xiphophorus* shows an almost schematically perfect nutritive relationship between parent and offspring. Within the peritoneal investment is a loose œdematous stroma, practically a lymph cavity, in which the follicles lie and from which the young ova derive nourishment before the establishment of their follicular circulation. The blood in the follicular capillaries seems to be separated from the cavity only by the endothelium and the squamous follicular epithelium. The embryo floats free in the follicular cavity, surrounded only by a very thin vitelline membrane. Beneath the epidermis of the yolk-sac is a dense bed of large blood vessels which function in the absorption of the yolk. This arrangement requires a molecule to penetrate four layers of cells and the vitelline membrane in passing from the blood stream of the parent to that of the embryo, a separation similar to that found in many mammalian placentas. Apparently *Xiphophorus* fails to become truly viviparous because of physiological rather than anatomical inadequacies.

The spent follicle of the teleosts is at times compared with the corpus luteum of the mammalia and is even so called by some authors. It is customary to limit the term to such ovulated follicles as contain that particular sort of irregular secretory cells known as lutein cells.

Cunningham (1897) describes the spent follicles of the plaice and the gray gurnard, both oviparous species. He finds the follicular epithelium granular and separated from the theca; it shows no signs of hypertrophy or proliferation. In late stages the follicle appears as a mass of connective tissue, which he thinks is most probably the much shrunken theca. No mention is made of any secretory activity of the follicle. But in the case of the weever, it is said to be full of cellular tissue. Wallace (1903), describing the spent follicle of the viviparous blenny, *Zoarces*, states that on rupturing the follicle collapses and becomes a papilla with only slight hypertrophy of the follicular epithelium. This papilla, according to Stuhlmann (1887), secretes the ovarian milk on which the embryos feed.

In *Xiphophorus* we find a hypertrophy of the spent follicle and of

the theca. The enlarged follicular cells do not look like lutein cells but much more like certain endometrial cells than like anything to be found in mammalian ovaries. The bulky secretion, which fills the follicle, cannot be of present nutritive use as the embryos are already delivered and the new follicles are not yet open to the lumen. It is unlikely that this represents only a retrogression of the follicle as it has not been described for oviparous teleosts nor does the follicle of disintegrating ova go through this phase. It conceivably might serve an endocrine function or serve to activate the sperm stored in the cells of the oviduct. In general *Xiphophorus* agrees with other teleosts in that its spent follicles cannot properly be termed corpora lutea.

Conclusions and Resumé

1. *Xiphophorus* is, in the anatomy of its female reproductive tract, very similar to *Gambusia* and *Glaridichthys*.

2. External dating of pregnancies is difficult, as body proportions, markings, and scale rings were found to be non-diagnostic.

3. The diameter of ova and length of embryos serve effectively to date ovaries which have been dissected.

4. Ovaries can also be assigned approximately to position from sections by noting the degree of differentiation of organs and tissues of the embryos.

5. Ova of varying size and yolk content can be found in the normal ovary at any stage of the cycle and are hence of no value in dating pregnancies.

6. As yolk accumulation commences, the height of the follicular cells decreases.

7. The follicular epithelium of a young ovum reaches its greatest height during the period of oil secretion before yolk granules appear.

8. After parturition the spent follicles collapse and hypertrophy, forming thick thecæ with a zone of capillaries and superficially a layer of tall columnar cells attached to each other only at the base.

9. The hypertrophied follicle, which is not considered directly comparable with the corpus luteum of the mammals, secretes and sloughs. The secretion appears as a crumpled mass of membranes and passes into the lumen of the ovary.

10. The organic content increases in the ovum until fertilization. From there on it becomes steadily less as it is used up by the growing embryo.

11. The ash content appears to increase throughout the entire period of gestation.

12. Water is absorbed in ever increasing amounts throughout the entire period of growth of ovum and embryo.

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THE ACTION OF THE CRUSTACEAN CHROMATOPHORE ACTIVATOR ON THE MELANOPHORES OF FISHES AND AMPHIBIANS

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In addition to inducing color changes in the crustaceans, the chromatophore activator of the crustacean eye-stalk also brings about contraction of fish melanophores (Koller and Meyer, 1930; Meyer, 1931), and expansion of amphibian melanophores (Perkins and Kropp, 1932). The range of occurrence and effectiveness of the eye hormone is very wide among the crustaceans, undoubtedly extending throughout the class where active chromatophores are present in the larval or adult stages (Kropp and Perkins, 1933). Concerning the action of the crustacean eye hormone on the melanophores of fishes, it is of interest to know the extent to which this substance is effective and the nature of the response by the fish melanophores. For this purpose several fishes were selected as representative of the local fauna, and sea-water extracts of various decapod eye-stalks were injected into them. The procedure consisted of adapting the fishes to white and to black backgrounds, noting the general coloring of the fish and the microscopic appearance of the melanophores. Measured amounts of the extract were injected into white-adapted and into black-adapted specimens and the fishes returned to their original black or white containers. Control animals in each group received a like injection of sea water.

The injections were made for the most part subcutaneously just ventral to the lateral line at the middle of the body, although in some cases it was given directly into the blood stream. Since no assay of the active substance that will permit the use of doses of known or comparable strength has yet been made, the procedure tentatively adopted was that previously outlined (Kropp and Perkins, 1933) of making the quantity of extract injected equivalent to a known number of eye-stalks. Two hundred eye-stalks were macerated in 1 cc. of sea water, boiled, centrifuged and the clear extract decanted off. The latter was then diluted with sea water to 2 cc., thus giving a solution in which each 0.01 cc. contained the active material of one eye-stalk.

Results

Anguilla rostrata

When about fifteen centimeters long this eel shows a large number of discrete melanophores that are easily observable with low magnification. It shows also the usual reactions to backgrounds,—melanophore expansion on black and contraction resulting in pallor on white backgrounds. The reaction, however, is relatively slow, for freshly captured animals may take as long as ten hours to show the characteristic white-adapted or black-adapted state, although some specimens may adapt in half the time. When they can be induced to remain motionless the rate of adaptation proceeds more speedily. The eels' response to extracts from the eye-stalks of white-adapted *Crago* was by no means sharp. Injections were made both subcutaneously and directly into the heart in different animals without any detectable difference in response. The minimum effective dose was 0.04 cc. of extract representing four eye-stalks. There were no lasting ill effects in most cases, but the eels never survived more than twenty hours if the quantity of fluid injected was increased to 0.05 cc. although uninjected animals could be maintained in the laboratory for indefinite periods.

At best there was a weak though definite response of the melanophores to the injection. In three to five minutes after injection, black-adapted eels showed a slight general melanophore contraction, somewhat more marked in the region around the place of injection. There was no apparent difference between animals receiving the injection subcutaneously and those receiving it directly in the blood stream. The effect obtained was always very transient and after ten to fifteen minutes the animals reverted to their original states.

Clupea harengus

In the black-adapted state the herring presents a fairly uniform dark metallic blue over the entire dorsum and sides. In the white-adapted state the blue gives way to a deep brown. We were not able to investigate the pigmentary conditions actually existing in the skin although it is apparent that some of the color phenomena that appear are due to interference. The changes from one state to another take place rapidly and never require more than one-half to one hour. The venter always retains its silvery white condition.

The minimum dose inducing an effect was ten eye-stalks (0.4 cc. of fluid used). In a black-adapted herring the effect of the extract was to bring on a local change at the place of injection. This change took the form of intensifying the blue characteristic of the black-adapted state.

It occurred within five minutes after the injection and after ten minutes this effect disappeared and no general effect was ever noted. In white-adapted animals, however, thirty minutes after the extract was injected the entire dorsum of the animal was blue-green and approximated closely the black-adapted state. The controls were injected with sea water, the black-adapted controls showing no effect, while the white-adapted controls took on a mottled blue-green state after one hour. Although the responses of both experimental and control white-adapted animals were alike in many respects, there were some striking differences,—namely, the slowness of the reaction in controls, one hour compared to one-half hour in the extract-injected animals, and the presence in controls of a pronounced mottling and never the uniform dark blue-green of the experimental animals. These differences in reaction are regarded as due to the presence of the chromatophore activator in the experimental group. Blue and blue-green color of sides and dorsum are assumed to be associated with a condition of melanophore expansion.

Pollachius virens

The ability of the pollock to adapt to its background is not highly developed. After several hours on a white background there was a suggestion of pallor over the back and sides, but marked differences in pigmentation were never observed. The injection of the extract also yielded no changes either in gross coloration or in the microscopic appearance of the melanophores. Doses of twenty eye-stalks failed to induce an effect in either black-adapted or white-adapted animals, whether the injections were made subcutaneously or by way of the caudal artery.

Scomber scombrus

The mackerel, unlike the pollock, shows a marked difference between the white-adapted and black-adapted states. In the former, black barring of back and sides is sharp and the spaces between bars are pale green-blue. In the latter, the intermediate spaces are blue-black to dark green or gray-black. The change from one condition to the other occurs readily in fifteen to twenty minutes. Like the pollock, however, this fish did not react to injections of extracts of as many as twenty eye-stalks.

Fundulus heteroclitus

For the minnow, *Fundulus*, extracts from eyes of *Homarus americanus* and *Pagurus longicarpus* were used in place of that from *Crago*. Mere puncture of the skin and underlying muscle while making an

injection usually resulted in the formation of a local spot of expanded melanophores due to the stimulation of a peripheral pigment motor nerve. Darkening due to such a stimulus, however, could always be detected promptly by the characteristic rapidity with which the effect occurred,—it was almost instantaneous. When a black-adapted *Fundulus* was injected with an extract of ten eyes of either *Homarus* or *Pagurus,* there usually occurred first the just mentioned local area of expanded melanophores. After five to fifteen minutes there supervened a condition in which the melanophores of this area contracted gradually until they were maximally contracted.

When a needle was simply thrust through the skin and muscle but no extract injected the same melanophore expansion resulted locally, but this lasted for five to twenty hours before again assuming the condition of the other body melanophores. The rapid contraction of the expanded melanophores of this region is to be ascribed, therefore, to the ability of the crustacean eye hormone to overcome the counter tendency of mechanico-nervous stimulation.

The area of contracted melanophores increased somewhat in size depending on the strength of extract injected. General pallor, however, was brought about only by means of a large dose which also induced serious ill effects and, in most cases, death. In one instance where an extract of twenty *Pagurus* eye-stalks was injected into a black-adapted *Fundulus* evidence of pallor first appeared after ten minutes. The pale patch at the place of injection then increased rapidly in size and the fish at the same time showed pronounced ill effects of the extract. It gasped, lay on its side, and its movements were distinctly abnormal. It survived for some fifteen hours after the injection, and during that time the pale area was extended to include the entire animal except for the dorsal and lateral surfaces of the head which remained black. In sub-lethal doses, then, the effect was to produce an area of contracted melanophores at the place of injection.

Before the first melanophore expansion due to nerve stimulation wore off there occurred normally a sharp melanophore contraction in the center of the area which spread peripherally until most of the melanophores in the entire area were contracted. It occurred in both black-adapted and white-adapted animals but was best seen in the latter. Here, the puncture produced an initial darkening, which soon gave way to pallor until there remained only a narrow band of expanded melanophores surrounding a region of maximally contracted ones. In most cases the contracted melanophores remained so for about two hours, but the area did not increase in size after the first fifteen minutes. With doses approaching the lethal the just described effect

was produced but the reacting melanophores then failed to revert to their expanded or stellate condition and acted subsequently as though paralyzed, and in some cases were known to have been killed.

Myoxocephalus octodecimspinosus

The long horn sculpin adapts very readily to backgrounds and also tends to adapt to the pattern of its background after the fashion of flat fishes. Injection of extract of ten eye-stalks of *Crago* into both black-adapted and white-adapted animals resulted in increased pallor all over the body. The marked local reactions characteristic of other species were here lacking in most specimens. The reaction set in five minutes after injection and disappeared after one hour. During the time that the extract was effective there was complete loss of ability to adapt to background and to pattern. The controls showed no response.

Limanda ferruginea

While the common dab adapts to backgrounds and to background patterns, it does not do so with the same ease and speed characteristic of other flat fishes. *Limanda* was able to survive a maximum dose of fifteen *Crago* eyes, and ten to fifteen minutes after the injection both black- and white-adapted animals exhibited a weak tendency towards melanophore contraction at widely separated parts of the body, but especially at the place of injection. This is in line with the results obtained by Miss Meyer (1931) with extracts of *Crangon* eyes on *Pleuronectes,* but she describes a more marked local reaction than we were able to obtain. It may well be, however, that the difference in results is due to a quantitatively inferior pigmentary reaction in *Limanda.* Unlike *Myoxocephalus,* the extract did not produce a loss of ability on the part of *Limanda* to adapt to its background pattern.

Rana clamitans

Previously the tadpoles of this frog were described as responding to injection of eye-stalk extracts from *Palæmonetes vulgaris* and *Callinectes sapidus* by melanophore expansion (Perkins and Kropp, 1932). In repeating those experiments with extracts from *Pagurus longicarpus* and *Homarus americanus* effects identical with those previously described were obtained. Extract of two eyes of either of these crustaceans was sufficient to induce melanophore expansion in white-adapted tadpoles five minutes after injection. Maximum expansion was reached after twenty to thirty minutes. Contraction began immediately and

the original white-adapted state was reached again one hour after the injection.

It was very striking to find, as we had in the case of *Callinectes*, that decapods which do not ordinarily show adaptive color changes or any chromatophore movements at all in response to backgrounds (*Homarus, Libinia, Pagurus*) all possess the active principle in their eye stalks. This is possibly a retention in the adult of a mechanism which is active in the larva where the chromatophores are mobile. The hormone of such crustaceans was found to be effective in producing chromatophore movements not only in other crustaceans, but, as shown in the present work, in fishes and amphibians as well.

TABLE I

Species receiving injection	Eye-Stalk Extract	
	Black-adapted	White-adapted
Anguilla rostrata	Slight contraction	No response or slight contraction
Clupea harengus	No response or doubtful expansion	Moderate expansion
Pollachius virens	No response	No response
Scomber scombrus	No response	No response
Fundulus heteroclitus	Extreme contraction	Moderate contraction
Myoxocephalus octodecimspinosus	Moderate contraction	Moderate contraction
Limanda ferruginea	Slight contraction	No response or slight contraction
Rana clamitans (tadpoles)	Moderate expansion	Extreme expansion

The extracts used were from eye-stalks of white-adapted *Crago vulgaris* in the cases of *Anguilla, Clupea, Pollachius, Myoxocephalus,* and *Limanda;* from *Pagurus longicarpus* and *Homarus americanus* in the cases of *Fundulus* and *Rana.* The terms "black-adapted" and "white-adapted" refer to the condition of the animal receiving the injected extract. The number of eye-stalks represented in the extract injected into each species is stated in the text. All controls received a corresponding amount of sea water, except *Rana* which received 0.7 per cent NaCl solution. All controls gave no response.

It is apparent from these results that the action of the crustacean eye hormone on fish melanophores is to bring about contraction. The case of *Clupea* is at first sight an exception, but we cannot yet regard it definitely so because of lack of accurate information as to the normal processes of pigment cell change involved in adaptation to backgrounds. All other reactions were clear-cut enough to be regarded as melanophore contraction due to the injection of the eye hormone. The response of *Rana clamitans* tadpoles also is confirmatory of previous work.

It had long been supposed that color changes in fishes were due entirely to the influence of the nervous system acting directly upon the chromatophores and that all observed chromatic phenomena were accountable for on that basis, by the function of the central, peripheral, and sympathetic nervous systems. This view was the more firmly held since the innervation of fish chromatophores had been demonstrated anatomically by Ballowitz in 1893. Melanophores of fishes were later found to respond to the secretions of the adrenal and pituitary glands, but the latter were not regarded as essentially necessary for normal pigmentary changes in fishes. In amphibians, on the other hand, it appeared that endocrine secretions controlled pigmentary changes in very large part, if not entirely; while among the reptiles both nervous and endocrine factors were believed to be the governing factors. With the establishment of the importance of humoral substances in amphibian color changes, reinvestigation of the situation in fishes revealed that while the central and sympathetic nervous systems undoubtedly play a large part in chromatophore reactions, all observed phenomena are by no means accounted for entirely on that basis. The evidence for this view has recently been reviewed by Parker (1932). The results here described lend weight to the view that humoral factors are involved in producing color changes in fishes, and that the response to a humoral substance from an invertebrate suggests a more general susceptibility to internal secretions than was first indicated by the reaction to their own or other vertebrate endocrines.

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SUPRAVITAL STUDIES ON THE COLORED CORPUSCLES OF SEVERAL MARINE INVERTEBRATES

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One of the most distinctive cytological features of the vertebrate erythrocyte is the characteristic reaction of its cytoplasm on exposure to appropriate concentrations of such vital dyes as neutral red and brilliant cresyl blue. As a result of this reaction the basophilic substance of the red blood cell is precipitated and aggregated to produce the so-called patterns of reticulation. The reticulation reaction occurs only in the erythrocytic series and seems to be definitely associated with the synthesis of the respiratory pigment, hemoglobin. The basophilic substance reaches a maximum in the early stages of hemoglobin formation and, as the concentration of hemoglobin within the differentiating erythrocyte increases, the basophilic or reticular material decreases. In the mature cells of the mammals this substance entirely disappears, while in other vertebrates it persists to varying degrees depending on the relative degree of differentiation attained by the erythrocytes.

In several of the invertebrates also, respiratory pigments, either hemoglobin or hemerythrin, are found within special cells of the circulating blood or body fluids. It seemed of interest, accordingly, to study the reaction of the invertebrate colored corpuscles to vital dyes in order to ascertain whether the reticulation reaction was specific for vertebrate red blood cells or was characteristic also of similar cells of invertebrates.

In this study four different species were utilized: *Phascolosoma gouldi* (Pourtalès), a gephyrean worm; *Glycera dibranchiata* Ehlers, a polychæte worm; *Thyone briareus* (Lesueur), a holothurian; and *Arca transversa* Say, a mussel. The corpuscles of *Phascolosoma* contain hemerythrin while those of the three others contain hemoglobin. Supravital preparations were made by the dry dye-film method (Dawson, 1932), using neutral red alone, neutral red in combination with Janus green B, and brilliant cresyl blue. Smears stained by Wright's method were also successfully prepared, but the initial application of the undiluted dye was prolonged to three or four minutes followed by a brief differentiation in the diluted dye.

PHASCOLOSOMA GOULDI

In this form the body cavity is relatively voluminous and contains a large amount of corpusculated fluid. This was readily drawn off from unanesthetized animals by means of a small hypodermic needle. The fluid is a pale rose color, due to the presence of numerous hemerythrin-containing cells suspended in the colorless plasma. The body fluid also contains numerous ova or spermatozoa. Since the activity of the spermatozoa makes supravital observations almost impossible, females were used in this study.

The corpuscles usually appear circular in outline and vary widely in diameter. When viewed on edge (Figs. 7, 8) they are seen to be relatively thick, slightly biconvex discs. The variation in size could not be correlated definitely with the degree of maturity of the cells since very large cells were observed with low concentrations of hemerythrin and vice versa. The variations in intensity of the pigmentation of the cells may also be due in part to varying degrees of reduction of the hemerythrin.

Owing to the density of the hemerythrin, distributed uniformly throughout the cell, the nucleus can rarely be distinguished in fresh, unstained preparations. Numerous fine granules, either colorless or light yellow, are seen in the interior of the cell. They may exhibit Brownian movement. Other large, colorless, more refractile vacuoles or globules may also be recognized. In dry-fixed smears stained by Wright's method the granules take a basophilic tint while the large vacuoles appear distorted and empty. Both bodies tend to be grouped about the small nucleus which occupies an eccentric position in the cell (Figs. 1 and 2). When stained supravitally with neutral red, the small granules are deep red while the vacuoles remain uncolored (Fig. 3). When Janus green B is added numerous granular mitochondria are seen distributed irregularly throughout the cytoplasm (Fig. 4).

The most striking staining patterns are produced with brilliant cresyl blue applied supravitally in concentrations high enough to stain the nucleus. The latter then appears light blue, the granules dark blue, and the vacuoles light pink. In addition a fine, flocculent, violet-colored material appears in the cytoplasm (Figs. 5 and 6). This eventually becomes aggregated into more or less definite strands or filaments resembling closely the patterns of reticulation which can be produced by the same staining method in the erythrocytes of vertebrates. The pink reaction of the large vacuoles with brilliant cresyl blue suggests that they are fat vacuoles. No induction or neo-formation of bodies stainable with neutral red was obtained even after long exposure to vital dyes.

Glycera dibranchiata

Glycera lacks a definite, organized circulatory apparatus and the hemolymph with the suspended corpuscles is readily obtained by aspiration directly from the body cavity. The corpuscles are relatively large and have the form of thin, slightly biconcave discs (Fig. 12). They are usually circular, occasionally oval, in outline. In fresh, unstained preparations the hemoglobin is seen to be uniformly distributed throughout the cell, usually in sufficiently high concentrations to completely mask the nucleus. Three different types of cytoplasmic inclusions can be identified without staining: several small yellow or orange granules, one or two large highly refractile globules, and numerous small vacuoles.

In dry-fixed smears stained by Wright's method a small, spherical, chromatic nucleus, usually eccentrically placed, may be seen. Occasional cells are bi-nucleated. The yellow and orange granules are basic in reaction and appear as black, brown, or blue bodies. The large refractile globules and small vacuoles are clear and uncolored.

After supravital staining with neutral red the colored granules appear deep red or brown. The large refractile bodies are light yellow and the small vacuoles are unstained (Fig. 9). With brilliant cresyl blue the colored granules are blue-black and the large globules pink. In addition to these inclusions recognizable in fresh cells, brilliant cresyl blue also reacts to produce two additional inclusions. Clusters of reddish-brown, needle-like crystals appear first; later a diffuse violet flocculation, which eventually changes into an irregular filamentous pattern, is also discernible (Fig. 11). The nature of the induced crystals was not determined. They may represent crystals of hemoglobin or hematin. The reddish-brown color is probably due to the addition of some component of the metachromatic brilliant cresyl blue to the original color of the crystals. The filamentous structures appear to be homologous with the reticulation patterns of vertebrate erythrocytes. Janus green B demonstrates numerous small, granular bodies scattered throughout the cell and these have been identified as mitochondria (Fig. 10). The large refractile globules are probably fat, but the nature and significance of the numerous small vacuoles within the corpuscles is problematic.

Thyone briareus

Hemolymph containing suspended corpuscles can be obtained directly from the perivisceral fluid of living animals by simple aspiration with a hypodermic needle. However, the corpuscles were found to be more numerous in the Polian vesicles (Van der Heyde, 1922; Kawamoto, 1927) and in most instances the cells studied were obtained from this

source. The neck of the vesicle was lightly grasped by a pair of flat forceps, the vesicle clipped free with scissors and transferred to the slide where it was ruptured by pricking with a needle.

The corpuscles are circular in outline and have the form of thin, slightly biconvex discs. In fresh, unstained cells the most conspicuous cytoplasmic inclusions are bright, yellowish-brown granules. They are relatively large and are found to vary in number. Occasionally they are entirely absent but usually one, two, or three, rarely four, are found. The hemoglobin is uniformly distributed throughout the cytoplasm with the exception of a narrow, peripheral zone, and the nucleus is usually invisible. The peripheral zone lacking hemoglobin appears alveolar in optical sections of the corpuscle (Fig. 16); with high focus the surface of the cell appears pebbled, being studded with small, closely packed vacuoles (Fig. 17).

After Wright's stain on dry-fixed smears a small, spherical nucleus is visible. It usually lies away from the center of the cell (Figs. 13 and 14). The colored granules are either unstained or appear brownish-black. The latter appearance is interpreted as evidence of a slight basophilia. Small colored spherules, lacking nuclei and staining with eosin, also occur rather frequently. They may or may not contain a colored granule (Fig. 15). They are probably identical with the minute corpuscles of Kawamoto (1927), who regards them as being devoid of hemoglobin. The cytoplasm of the corpuscles stains intensely with eosin except for a narrow, peripheral zone. This represents the alveolar border seen in fresh cells; in dry-fixed cells the alveolar feature was not evident. A similar unstained surface zone was described by Kawamoto (1927) for the corpuscles of *Caudina chilensis*.

The colored cytoplasmic granules stain intensely with both neutral red and brilliant cresyl blue and in old preparations show active Brownian movement. Mitochondria are readily demonstrated with Janus green B. They appear granular at first, but as the preparation ages and Brownian activity becomes more marked they are seen to be short rods oriented at right angles to the flattened surfaces of the cell. However, the rod-like nature is apparent only when their normal orientation is disturbed by Brownian movement. As in *Phascolosoma* and *Glycera*, high concentrations of brilliant cresyl blue cause a definite filamentous structure to appear throughout the cytoplasm (Fig. 18). This also has the characteristic reddish-violet tint. No fat globules or other vacuoles were observed in the corpuscles of *Thyone*.

Arca transversa

When the valves of a specimen of *Arca* are separated there is usually a considerable collection of red fluid to be found in the mantle

cavity. This is described by Cuénot (1891) as being forced out of the circulation by the contraction of the large sinus of the foot. Hemolymph may also be obtained by breaking through the shell in the region of the umbo and aspirating fluid directly from the cardiac region.

The corpuscles of *Arca* are relatively small and either circular or oval in outline. When viewed on edge they are seen to be extremely thin, slightly biconvex discs. The hemoglobin is uniformly distributed throughout the cytoplasm and the nucleus can rarely be distinguished in fresh cells. The only visible cellular inclusions in unstained preparations are numerous yellow-brown granules of irregular size and shape which exhibit varying degrees of Brownian activity.

After Wright's stain a small, spherical nucleus, generally eccentric, is visible (Figs. 19 and 20). The colored granules are conspicuous on the eosinophilic background and usually do not react appreciably with any component of Wright's stain. In some preparations they appear brownish-black, probably indicative of a slight degree of basophilia. The granules stain deeply with supravital applications of neutral red. They also appear to lose their irregular outline and become spherical on long exposure to the dye. After assuming the spherical form they may increase in size, but no new staining loci appear within the cell (Fig. 21). It is not entirely clear whether the granules actually change form or are completely obscured by a densely stained vacuole which develops about them. In old preparations in which the corpuscles have lost much of their hemoglobin the stainable bodies are less densely colored and a small refractile center can be distinguished within them, suggesting that the material of the granule has partially dissolved in the fluid of the enveloping vacuole.

Janus green B brings out a variable number of small, perinuclear bodies which appear either as granules or short rods. These are interpreted as mitochondria (Fig. 22). With brilliant cresyl blue the irregular granules take a deep blue stain and round up as after treatment with neutral red. In the early stages of staining a fine, granular material is seen scattered throughout the cell (Fig. 23) but later this is aggregated into rather indefinite, irregular filaments (Fig. 24).

The corpuscles of *Arca* are quite sensitive to changes in the osmotic pressure of the medium in which they are suspended (Sato, 1931), and typical crenation, of the thorn-apple type encountered in vertebrate erythrocytes, is readily produced (Fig. 25). Crenated corpuscles frequently rupture and one or several balloon-like formations may appear on their surfaces (Figs. 26 and 27). So far as could be determined, only the more fluid contents of the corpuscle, including the hemoglobin, enter the bud-like protuberances, the colored granules and nuclei al-

PLATE 1

All figures were outlined at the same magnification (1200 diameters) by means of a camera lucida. Cells were selected to show the range in size within each species.

Phascolosoma gouldi:

1 and 2. Corpuscles from a dry-fixed film stained by Wright's method showing eccentric nucleus, basophilic cytoplasmic bodies, and unstained globules or vacuoles.

3. Fresh corpuscle stained supravitally with neutral red. The basophilic bodies (black) stain intensely while the clear globules are uncolored.

4. Fresh corpuscle stained supravitally with Janus green B showing mitochondria.

5 and 6. Fresh corpuscles stained supravitally with brilliant cresyl blue showing the basophilic bodies (black) stained deep blue, the clear globules stained pink, and the reticulation filaments stained violet.

7 and 8. Fresh corpuscles seen on edge.

Glycera dibranchiata:

9. Fresh corpuscle stained supravitally with neutral red. Bodies (black) which were yellow or orange in unstained cells were colored deep red. Large clear refractile globules (gray) stained yellow. Numerous small clear vacuoles were uncolored.

10. Fresh corpuscle stained supravitally with Janus green B showing only mitochondria.

11. Fresh cell stained supravitally with brilliant cresyl blue. The nucleus was colored light blue. The small bodies (black) were deep blue, the two larger globules (gray) pink, and the reticulation filaments violet. Violet needle-like crystals also appear after treatment with brilliant cresyl blue.

12. Fresh corpuscle seen on edge.

Thyone briareus:

13, 14, and 15. Corpuscles from a dry-fixed film stained by Wright's method. Refractile bodies, colorless, yellow or brown in fresh cells, give a basic reaction. Fig. 15 shows a small, non-nucleated corpuscle.

16. Fresh corpuscle stained supravitally with neutral red and Janus green B showing the refractile bodies (black) which were deep red and the mitochondria (gray). This corpuscle is shown in optical section to demonstrate the vacuolated or alveolar periphery.

17. Fresh corpuscle at high focus to show the pebbled or vacuolated appearance of the surface of the cell.

18. Fresh corpuscle stained supravitally with brilliant cresyl blue: nucleus, light blue; nucleolus, deep blue; refractile bodies, purple or deep blue, and the reticulation filaments, violet.

Arca transversa:

19 and 20. Corpuscles from a dry-fixed film stained by Wright's method. The cells contain irregular, yellow or brown granules which give a slightly basic reaction.

21. Fresh corpuscle stained supravitally with neutral red. The refractile bodies stain intensely with neutral red and become spherical in form.

22. Fresh corpuscle stained with Janus green B showing only the mitochondria.

23. Fresh corpuscle stained with brilliant cresyl blue showing the early flocculent aggregations induced by the action of the dye.

24. Fresh corpuscle stained with brilliant cresyl blue showing the later filamentous form of the reticulation substance. The refractile granules stain deep blue and round up as with neutral red.

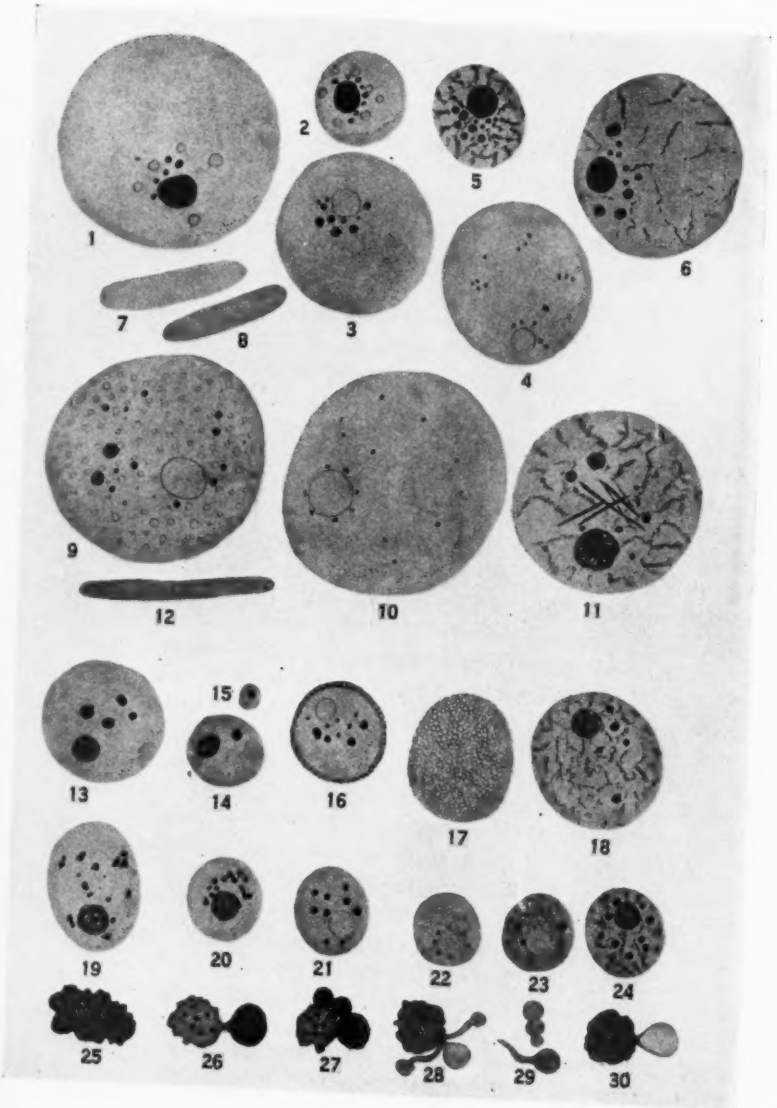
25. Crenated corpuscle from a fresh preparation.

26. Rupture of a crenated corpuscle. The nucleus and refractile granules remain in the body of the cell while the hemoglobin enters the balloon-like extension.

27. Same as above but with three such extensions.

28, 29, and 30. Similar cells after 18 hours in a sealed preparation. Most of the hemoglobin has been lost, revealing the delicate investing membrane of the cellular extensions.

PLATE I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ways remaining in the main portion of the cell. After supravital staining with brilliant cresyl blue the reticulation reaction is obtained only in the buds. In preparations allowed to stand overnight the hemoglobin is usually lost from the buds and a delicate, investing membrane is then visible (Fig. 30). The buds may also change in form, appearing as small knobs attached to the main portion of the cell by slender stalks (Fig. 28). They then exhibit Brownian activity and in many instances become separated from the corpuscles (Fig. 29) to float freely in the hemolymph.

Discussion

It is frequently stated in textbooks that among the invertebrates there are no cells which are exactly comparable to the erythrocytes of the vertebrates. The invertebrate colored corpuscles are described as nucleated, ameboid cells resembling white corpuscles, each cell containing a small globule of pigment in its cytoplasm. Such statements are in direct opposition to all published descriptions of the invertebrate cells which carry respiratory pigments (Cuénot, 1891; Romieu, 1923, and others). These cells are usually nucleated, but are not ameboid, and the respiratory pigment is uniformly distributed throughout the cytoplasm. They are invested by a definite membrane, which appears to be elastic since the corpuscles readily recover typical form after distortion. They hemolyse in the same manner as vertebrate erythrocytes and ordinarily respond similarly to changes in osmotic pressure, exhibiting characteristic swelling and crenation.

In the four species examined at Woods Hole all the corpuscles are circular or oval in outline and have a disc-like form, but the relative thickness of the cells differs in each species. The size of the cells is more variable within the species than in the vertebrates, but the dimensional proportions of the cells of each species are nearly constant. Spherical corpuscles have been described in *Thallasema mellita* by Abbott (1913) and in *Urechis caupo* by Baumberger and Michaelis (1931).

Non-nucleated corpuscles have been reported for only one species of worm, *Magelona papillicornis*. In this form the respiratory pigment is hemerythrin. The early observations of McIntosh (1878) and Benham (1896) regarding the uniform absence of the nucleus have been confirmed by the more recent studies of Romieu (1923). Small, non-nucleated spherules were encountered in the hemolymph of *Thyone* in both fresh preparations and stained smears. Many contained characteristic, colored, granular inclusions and the plastids appeared to be similar in constitution to the larger nucleated elements. No evidence regarding their origin was obtained. Presumably they arise by a process of budding.

The cytoplasmic inclusions of the invertebrate corpuscles are of several kinds: clear vacuoles of undetermined nature, fat globules, and granules. The granular inclusions appear to be characteristic of almost all invertebrate corpuscles whether the respiratory pigment be hemoglobin or hemerythrin. They react readily with vital dyes and may exhibit varying degrees of basophilia. In fresh unstained cells they are readily recognized by their high refractive index and are usually yellow or brown in color. Their nature was not determined. In the literature they are frequently referred to as excretion granules. In *Thallasema*, Abbott (1913) obtained a positive test for iron in these granules, and Baumberger and Michaelis (1931) identified the brown granular pigment in the corpuscle of *Urechis* as hematin.

Vital dyes have not been used extensively in the study of the invertebrate corpuscles, and in most instances when they have been applied the concentration was only sufficient to stain the preformed granular inclusions. However, Romieu (1923) found that in *Notomastus* vital staining with high concentrations of dye caused the appearance of a coarse-meshed network whose branches tended to radiate from the nucleus to the periphery of the cell. He also obtained a somewhat similar pattern in the corpuscles of *Glycera tesselata* after hemolysis and suggested that it might correspond to the substantia granulo-filamentosa (reticulation substance) of the vertebrate erythrocyte.

The present observations show that in the four species studied, patterns resembling the vertebrate reticulation can be obtained by the application of brilliant cresyl blue in suitable concentrations. This staining reaction may be obtained without causing hemolysis but the nucleus is always stained. Similarity of staining reaction and of morphology of the intracellular patterns are admittedly inadequate criteria to establish the identity of the substances occurring in the invertebrate and vertebrate corpuscles, but the uniformity of the reaction suggests that in the elaboration of an intracellular respiratory pigment, whether hemoglobin or hemerythrin, the synthetic processes are fundamentally alike.

Summary

Supravital studies of the colored corpuscles of the hemolymph or body fluid of four species of invertebrates, *Phascolosoma gouldi*, *Glycera dibranchiata*, *Thyone briareus*, and *Arca transversa* are recorded. The corpuscles of *Phascolosoma* contain hemerythrin; the others, hemoglobin.

All the corpuscles are disc-like in form and the respiratory pigment is uniformly distributed throughout the cell.

The corpuscles are usually nucleated, but in *Thyone* a variable number of small, non-nucleated spherules were noted.

Mitochondria were present in all cells. Other cytoplasmic inclusions consisted of vacuoles of undetermined nature, fat globules and refractile granules, either colorless, yellow or brown. The granules react readily with vital dyes and are slightly basophilic. Their composition was not determined but it is suggested that they are derivatives of the respiratory pigment.

Rather extensive patterns of reticulation, resembling those of vertebrate erythrocytes, appear in the corpuscles of all four species on exposure to suitable concentrations of brilliant cresyl blue.

The corpuscles of *Thyone* are characterized by a peculiar alveolar zone immediately beneath the cell membrane.

The corpuscles of *Arca* frequently rupture when crenated and much of the fluid content, including the hemoglobin, enters the thin-walled, balloon-like extensions found on the surfaces of the cells. The nuclei and specific granules remain in the main body of the cell.

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MATING IN LIMULUS POLYPHEMUS[1]

CHARLES MARC POMERAT

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Homogamy, the tendency of like to mate with like, has been definitely established for several plants and animals. A general account especially from the plant side has been given by D. F. Jones (1928). Willoughby and Pomerat (1932) have summarized the zoölogical findings. In a study by H. E. Jones (1929) and one by Schiller (1932), the literature dealing with homogamy in psychic characters in man has been reviewed.

The factors involved in such selection may be simple morphological characteristics depending upon the mechanics of the mating act. The liberation of specific substances preceding mating may also play a rôle by stimulating olfactory receptors of the opposite sex. Complex behaviour, degree and extent of activity, and more specifically preliminary sex behaviour, may also be important in the process of selection. This involved mating behaviour in man is generally referred to as psychic, and is usually described in psychological terminology. Homogamy in man may depend largely upon psychic factors, while activity (as a function of age) may be responsible for the relatively high degree of correlation in toads (Willoughby and Pomerat, 1932). Mechanical adjustment of the structures involved in the mating act may afford the simplest explanation for selective coupling since, a priori, it would seem that successful mating is possible only within certain limits of form which in turn are conditioned by (1) the complexity of the coupling, *i.e.*, the number and character of the points of contact and (2) the degree of mobility of the body to allow for adjustment. To these variables a third might be added—relative activity and physical vigor —since successful mating may require pursuit and capture, display mechanisms, and (at least in the cases where amplexus is involved) that, for a period, the participants be attached to each other.

In a previous paper (Pomerat, 1932) it was pointed out that results obtained from the study of homogamy in invertebrates were not, contrary to previous opinion (Willoughby and Pomerat, 1932), consistent among themselves. Workers in this field have tended to suppose that

[1] This investigation was aided by a scholarship from the Collecting Net.

cœfficients of correlation for homogamy might be high for organisms having relatively inflexible exo-skeletons. This would seem especially possible, from a mechanical standpoint, for species practicing internal fertilization where at least two points of contact are involved. This, however, was not found to be the case in a study of the Japanese beetle, *Popillia japonica* Newm. (Pomerat, 1932), in which the usual dorsoventral position is taken, with clasping by the male in addition to the genital contact. It was not found possible to isolate the chief mechanical structures upon which amplexus is dependent, unless the structures studied, namely the four tibia and tarsi, actually are critical in the process. The central tendency of the distribution of cœfficients derived from eight different measures was very slightly positive in the Japanese beetle. In view of this unexpected finding it was thought advisable to examine the mating behaviour of other arthropods. The species selected for this study, the horse-shoe crab, *Limulus polyphemus* L., is of particular interest since it represents a form in which copulation involves amplexus but with no intromission, so that there is only one pair of contact points involved in the coupling, *i.e.*, clasping without genital contact. It was conjectured that the removal of the partial restriction imposed by genital contact might result in an increased range of choice and consequent lowered cœfficients. A significant difference in the latter, as between the two types of mate-union, might thus afford some support for the theory of mechanical determination of homogamy. In addition, *Limulus* is the largest invertebrate which has been studied thus far from the standpoint of homogamy. Considering technical difficulties involved in obtaining biometric data, the horse-shoe crab owing to its large size should form a relatively more reliable source of information than the arthropods heretofore examined.

Materials and Methods

One hundred pairs of the horse-shoe crab, *Limulus polyphemus* L., were taken in the vicinity of Woods Hole, Massachusetts. The largest group (49 pairs) was found along the sandy beaches on either side of Penzance Point. All measurements were taken between June 1 and June 5, 1932. Mated *Limuli* are most often found after sun-down, at high-tide, in shallow water along protected sandy beaches. According to Mr. George Gray of the Marine Biological Laboratory Museum at Woods Hole, the height of mating activity is reached during the full moon late in May or early in June. Kingsley, writing in 1892, stated that he had never found this correlation. Unfortunately the writer was unable to confirm either view.

By means of modified pincers of the second pair of feet the male

grasps the hinder half of the abdomen of the female and trails along in shallow water. Locomotion of mated pairs being relatively slow makes the collection of animals easy.

All measurements were taken in inches to the nearest sixteenth. These data were later converted entirely to sixteenths. This system

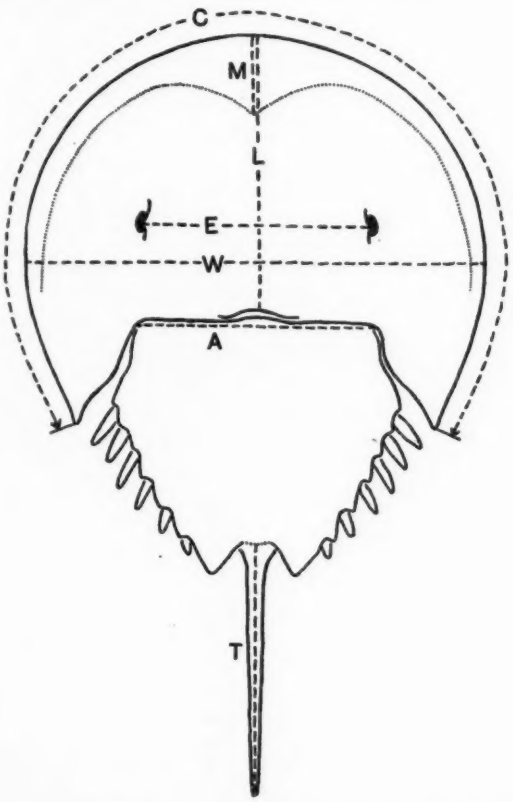

FIG. 1. Diagram of the dorsal aspect of *Limulus polyphemus* illustrating measurements taken.

The outline of the animal is shown by unbroken lines (eyes in solid black); dotted lines indicate structures of the ventral surface; interrupted bars show the course of the seven measurements.

was one of expediency, since at the time of collection it was impossible to procure a steel tape marked in metric measure for use on curved structures.

TABLE I

W	90	95	100	105	110	115	120	125	130	135	140	145	150	155
m	1			1	6	9	26	21	16	3	12	3	1	
f									1		3	6	5	5

	160	165	170	175	180	185	190	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m								99	125.1	9.5	7.6		
f	26	13	11	17	7	1	4	99	164.6	12.0	7.3	25.8	1.6

L	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135
m	1		1	7	25	23	20	19	3	1					
f									1	3	4	16	15	18	20

	140	145	150	155	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m					100	131.2	7.5	5.7		
f	12	8	1	1	100	89.9	9.5	5.0	34.1	1.6

M	20	22	24	26	28	30	32	34	36	38	40	42	44	N	M	σ
m	1	2	17	32	27	15	6							100	27.02	2.4
f						2	2	6	29	20	27	7	7	100	38.14	3.0

	V	d_M/σ_d	d_σ/σ_d
m	8.8		
f	7.8	34.1	1.6

C	200	205	210	215	220	225	230	235	240	245	250	255	260	265
m	2	8	14	7			1							1
f														
	270	275	280	285	290	295	300	305	310	315	320	325	330	335
m		2	3	2	6	8	5				6	7	4	7
f													1	
	340	345	350	355	360	365	370	375	380	385	390	395	400	405
m	3	2	4	5	1	1	1							
f			2	2	1	1	1	2	4	4	9	3	4	3
	410	415	420	425	430	435	440	445	450	455	460	465	470	475
m														
f	13	7	1	9	8	5	5	5	3	2	2		1	1

	480	485	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m			100	283.8	55.0	12.3		
f		1	100	412.85	29.0	7.0	20.9	4.2

E	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125
m	1	1	15	14	33	26	10								
f						1	3	8	21	17	24	18	7		1

	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	100	74.75	6.5	8.7		
f	100	101.8	8.0	7.8	26.2	1.4

T	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135
m	1		2	1	1	4		9	9	10	13	7	11	6	3
f								2		3	5	3	1	4	8
	140	145	150	155	160	165	170	175	180	185	190	195	200	N	
m	4	1		1										83	
f	13	10	8	6	7	7	1	3			1		1	83	

	M	σ	V	d_M/σ_d	d_σ/σ_d
m	113.75	16	14.0		
f	144.00	19	13.2	11.0	1.1

TABLE I (*Continued*)

A	50	55	60	65	70	75	80	85	90	95	100	105	110	115	120	N
m	1	2	11	39	30	12	5									100
f				1		3	10	16	20	31	13	3	2		1	100

	M	σ	V	d_M/σ_d	d_σ/σ_d
m	67.55	5.5	8.1		
f	91.50	8.0	8.7	24.7	2.5

Seven measurements were taken and recorded in the field. These were grouped and designated (see Fig. 1) as follows:

I. Circumferential Measure.
   C—curved distance around cephalothorax from one posterior marginal spine to the other.

II. Median Sagittal Measures.
   M—length of median widening of the rim of ventral carapace;
   L—length of cephalothorax along its curvature;
   T—telson length, ventral aspect measured from the ridge distal to muscular attachment of the base to the tip.

III. Width Measurements.
   E—curved distance between the median edge of the compound eyes;
   W—greatest width of the cephalothorax taken straight across the ventral side;
   A—width of abdomen at the cephalothoracic junction.

The distribution of direct measures is given in Table I.

It is evident that the females are significantly larger and cover a wider range of sizes than the males. It should be noted that the scatter of measurements for the character *C* (circumference of the cephalothorax) in the male shows a trimodality with peaks in the vicinity of 210, 290, and 335. Of the following coefficients, those in which this measure is involved are slightly incomparable with the others for this reason.

One hundred and four Pearson product moment correlations were obtained from 1400 measurements taken on 100 pairs of mated *Limuli*. These will be considered in the following order: (1) direct and cross-correlation, (2) self correlations, (3) quotient correlations.[2]

Direct and Cross Correlations

The results of correlations of these measurements are given in Table II.

Direct correlations range from —0.005 to 0.151. The lowest value obtained (—0.005) existed between the abdominal width of mated pairs. This was the lowest value found for the entire series. The

[2] It is a pleasure to acknowledge indispensable assistance from Miss Edith Fishman, and from Mr. James Sell.

highest direct correlation value (0.151) was found for the cephalothoracic width of mates.

Cross correlation values range from -0.01 to 0.246; the former value existing between the median width of the rim of the ventral carapace of the female and the male cephalothoracic circumference, while the latter, the highest value for both direct and cross correlations, was found for the relation between the telson length of the female and the cephalothoracic width of the male. It must be noted, however, that the telson length cannot be absolutely relied upon since it was frequently found that the telson was eroded to a varying degree, and in a few cases it was broken. These accidents arise from the horse-shoe crab's habit of burrowing in sandy and muddy shores. The crab first arches its body by bending it at the joint between the cephalothorax and the abdomen, draws the sixth pair of legs forward and then extends the body, pushing with the legs against the firm hold of the telson. In view of the unreliability of the measurement, *T*, it is interesting to note that the next highest measurement (0.202) is found between the cephalothoracic circumference of the female and the median widening of the rim of the ventral carapace in the male.

TABLE II

Direct and Cross Correlations Obtained for Seven Characters Measured on 100 Mated Pairs of Limulus polyphemus L.

		Males							
		W	L	M	C	E	T	A	
Females	W	0.151	0.104	0.118	0.036	0.083	0.044	0.087	
	L	0.107	0.147	0.118	0.090	0.101	0.073	0.079	
	M	0.084	0.081	0.111	−0.010	0.083	0.030	0.090	
	C	0.136	0.156	0.202	0.103	0.128	0.042	0.083	$M_r = 0.10$
	E	0.161	0.121	0.182	0.044	0.101	0.020	0.062	
	T	0.246	0.153	0.150	0.004	0.120	0.081	0.080	
	A	0.076	0.022	0.150	0.020	0.023	0.001	−0.005	

The abdominal width measure *A* in the female appears to influence low correlations most frequently; five cases with values of less than 0.025 occurring in the series. The greatest cephalothoracic width *W* and the median sagittal width of the ventral carapace *M* in the male seem to influence high correlations; four values of more than 0.130 being found for each in the series. The mean value for 49 direct and cross correlations is approximately 0.100. With a probable error of ± 0.06 it is quite possible that the values obtained are chance results. Measurements on at least 500 individuals would be necessary to lower the probable error enough to obtain fixed values. The correlation values obtained, however, strongly suggest that homogamy as regards size in

Limulus polyphemus L. is practically non-existent. If a slight correlation should turn out to be real and not merely due to sampling, it might conceivably be explained on the basis of the slight limitations imposed by the adjustment of the male clasping claw to the female carapace.

Self Correlations

This designation is used for correlations between different measures of the same individual. See Table III.

The means of the 42 self correlations possible with seven measures were 0.65 for the males and 0.78 for the females. The highest coefficients (0.97 for both sexes) were those expressing the relationship between interocular width and cephalothoracic length; the lowest for the male (0.23) was between the cephalothoracic circumference and the abdominal width, and for the female (0.51) that between the telson length and the greatest distance across the rim of the ventral carapace.

The influence of the circumference of the cephalothorax measure on

TABLE III

Coefficients for Self Correlations

	E–L	*L–A*	*L–W*	*A–W*	*E–A*	*E–W*	*N–W*	*M–E*	*M–L*	*M–A*	*T–A*
m	0.97	0.89	0.88	0.85	0.84	0.83	0.77	0.73	0.72	0.70	0.59
f	0.97	0.84	0.88	0.84	0.89	0.85	0.82	0.76	0.82	0.79	0.49
	T–L	*T–E*	*T–W*	*C–W*	*C–L*	*M–C*	*T–M*	*E–C*	*T–C*	*C–A*	Mean
m	0.59	0.57	0.55	0.52	0.51	0.51	0.48	0.45	0.33	0.23	0.65
f	0.56	0.52	0.62	0.94	0.96	0.82	0.51	0.91	0.65	0.95	0.78

other characters seems to operate in opposite directions in the male and in the female. The cephalothoracic measurement of the female, for the most part, combines with every other measurement to give high correlations, whereas the cephalothoracic measurement of the male never does; for example, self correlation *C–A*, where the male gives 0.23 and the female 0.95. It is significant that the largest measure taken, the circumference of the carapace, is related with every other characteristic studied to give high self correlation (consistency of proportion) in the female, while in the male this tendency is relatively negligible.

In the seven characteristics studied the female was found to show the greater range in size, the greater variability, and a more marked consistency of form. Low self correlations between telson length and all other measures in both male and female further serve to indicate that the telson measure has a prominent chance error in it. If self

correlation values are eliminated when they contain the telson measure the means are raised noticeably; for the male the M_r becomes 0.69 and for the female 0.87. This greater variability and higher self correlation in the female has also been found for the toad by Willoughby and Pomerat (1932) and for the Japanese beetle by Pomerat (1932).

TABLE IV

C/W	150	155	160	165	170	175	180	185	190	195	200	205	210
m	2	1	7	14	7	1	1						
f													
	215	220	225	230	235	240	245	250	255	260	265	270	N
m		2		1	6	16	12	16	5	6	1	1	99
f				1	4	19	28	31	14	1		1	99

	M	σ	V	d_M/σ_d	d_σ/σ_d
m	218.85	39	13.2		
f	246.85	6	2.7	7.1	8.4

L/W	64	66	68	70	72	74	76	78	80	82	84	86	88	90	92	N
m	2	1	5	26	29	16	14	5								98
f					1	11	25	19	25	9	6			1	1	98

	M	σ	V	d_M/σ_d	d_σ/σ_d
m	72.24	2.8	3.8		
f	78.48	3.2	4.0	14.8	0.95

E/W	52	54	56	58	60	62	64	66	68	N	M	σ	V	d_M/σ_d
m	1	3	12	29	23	20	10		1	99	59.54	2.8	4.7	
f			2	16	27	25	21	5	3	99	61.50	2.6	4.2	5.1

	d_σ/σ_d
m	
f	.52

A/W	46	48	50	52	54	56	58	60	62	64	66	68	N	M	σ
m		3	4	18	39	26	6	2		1			99	56.28	2.4
f	1		3	12	26	39	13	3		1		1	99	53.30	2.8

	V	d_M/σ_d	d_σ/σ_d
m	4.2		
f	5.2	8.2	1.1

T/W	51	54	57	60	63	66	69	72	75	78	81	84	87	90	93	96	99
m	1			1	1	2	1		5	2	5	6	7	12	10	6	14
f						2	1	2	8	10	7	8	10	10	10	2	8

	102	105	108	111	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	3	3	1	2	82	89.61	11.1	12.3		
f	3	1			82	86.10	9.0	10.4	2.3	1.4

Quotient Correlations

Quotients obtained by dividing one character by another indicating other sex differences are as given in Table IV.

Quotient or morphological indices were correlated directly in fourteen cases. See Table V.

TABLE V

Quotient Correlations (Morphological Indices)

M/C	0.16	E/A	0.05	M/E	−0.07
C/W	0.14	E/W	0.03	T/W	−0.08
L/W	0.11	L/E	0.01	M/A	−0.11
L/M	0.11	T/L	−0.01	C/A	−0.225
C/E	0.08	A/W	−0.01	M_r	0.086

Quotient values were treated to obtain correlations for form. The consistently low values obtained gave a mean value of 0.086, which would suggest that if there were a real, though slight, homogamy in *Limulus*, this would depend upon size rather than form.

DISCUSSION

A biometric study of one hundred mated pairs of the horse-shoe crab, *Limulus polyphemus* L., was made to test the theory of mechanical determination of homogamy.

TABLE VI

Correlation Coefficients for Homogamy in Invertebrates (Averages)

Species	Character	r	Author
Paramecium	Length	0.61	Pearl (1907)
	Breadth	0.30	
	Index (Breadth/Length)	0.43	
	Length (wild cultures)	0.38	Jennings (1911)
	" (two species)	0.94	
	" (pure races)	0.23	
	Breadth (wild cultures)	0.33	
Chilodon uncinatus	Length	0.40	Enriques (1908)
Chromodoris zebra	Mantle length	0.52	Crozier (1918)
	Total length	0.61	
Gammarus locusta	Length	0.91	Crozier and Snyder (1923)
	Length	0.80	Crozier (1918)
Dikerogammarus fasciatus	Length	0.69	Crozier and Snyder (1923)
Popillia japonica	Length	0.19	Pomerat (1932)
	Thoracic Width	0.18	

Coefficients of correlation have been found to be high for species in which precise apposition of contact points is necessary for copulation, as indicated by Table VI.

Conjugation in the protozoans, *Paramecium* and *Chilodon;* hermaphroditism in *Chromodoris;* and the complexity of mating in gammarids, as described by Holmes (1903), suggest that it may be possible to account for the high correlations obtained on the basis that the mechanical precision involved does not permit coupling of individuals of widely different sizes.

The findings for the Japanese beetle, *Popillia japonica,* do not tend to uphold this view, unless it can be considered that a greater degree of adjustment of contact points is possible than might a priori be expected.

The findings in *Limulus polyphemus* are in keeping with the mechanical theory, on the basis that only one point of contact is involved. The range of choice is, therefore, markedly increased and the coefficient of homogamy proportionately lowered.

There is some evidence then that mating in certain invertebrates rests at least in part upon a mechanical process.

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PHENOMENON OF REGENERATION IN EVERTED HYDRA

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INTRODUCTION AND HISTORICAL

The first worker to observe that a hydra turned inside out might regenerate was the Swiss priest, Alexander Trembley (1744). Trembley maintained that the ectoderm, which in the turned animal lines the enteric cavity, came to take over the functions of the endoderm; while the endoderm, which was outside, assumed the functions of the ectoderm. Nussbaum (1887, 1891) repeated Trembley's experiment, but came to the conclusion that, instead of reversal of function, migration of the layers took place. He described sliding of the ectoderm over the endoderm, beginning at the mouth and a pore in the base, and continuing until the entire ectoderm had reached its outside position. Ischikawa (1890) undertook the same problem and obtained the same results as Nussbaum, but he explained them much more plausibly. Both Nussbaum and Ischikawa criticized Trembley for his manner of proving that the everted animals remained everted and did not revert themselves. The present paper deals with the changes occurring in ectoderm and endoderm after the position of these two layers has been reversed by turning. The actual explanation of the regeneration phenomenon is quite different from any previously offered.

MATERIALS AND METHODS

For this work specimens of three common species, *Hydra viridissima* Pallas (=*viridis* L.), *H. vulgaris* Pallas (=*grisea* L.) and *H. oligactis* Pallas (=*fusca* L.), have been used, each in the manner in which it was best suited. The first proved to be best for studies on the living material; the latter two were better for the study of fixed and stained material.

The method of turning was very simple. A hydra in a small dish with a few drops of water under a binocular microscope was stimulated to contract by careful prodding with a needle. When it had fully

[1] The writer feels greatly indebted to Dr. S. H. Derickson and Dr. V. Earl Light of Lebanon Valley College, and to Dr. Elery R. Becker of Iowa State College for guidance and criticism concerning the problem and preparation of the manuscript.

contracted, its anterior end was placed on the bottom of the culture dish, care being taken that its tentacles radiated from the hypostome in the normal manner. Then, very carefully, the basal disk was pushed with a fine needle until it came in contact with the bottom of the dish through the mouth of the hydra. In order to accomplish this, a number of successive movements of the needle, so as to force the mouth open and the body through it down against the glass, was necessary. Then the mouth portion was carefully manipulated with another needle so as to force it to come back up against the first-mentioned needle. The hydra was now turned and rested in an inverted position on the point of one of the needles. It was then very carefully pushed off, and more water added.

Animals to be fixed were allowed to remain in the inverted condition for a predetermined length of time, and then killed with warm corrosive acetic poured onto them in a stream from a fine pipette, beginning at the base. In this way the hydras were killed in an extended condition. This material was stained *in toto* with borax carmine and sectioned as thin as 5 and 10 μ.

Results

In the earlier work previously mentioned it was found that hydras which have been turned fall into three distinct classes according to their subsequent behavior: First, those which are not able to adjust themselves to their new situation, and so almost immediately undergo "depression," or slow contraction accompanied by disintegration, and finally death, unless re-turning and recovery supervene; second, those which attempt to re-turn themselves; and third, those which remain turned and regenerate.

It has been found that, out of a group of 60 hydras taken from their native pool and turned on March 19, 1931, 21 underwent "depression," 18 re-turned themselves, and 21 remained turned and readjusted themselves.

After having observed approximately 300 hydras turn themselves back to their normal condition, the writer can affirm that it took place nearly every time in the manner originally described by Trembley. The everted hydra has its tentacles extending out of its mouth in a group. It will, of course, be realized that hydras in such a condition would generally be contracted to the fullest extent. This results in the mouth being pulled closely around the tentacles which pass out through it. The inside mouth comes to be on the outside, and the hollows of the tentacles, which normally are connected with the enteric cavity, can be seen to open on the outside. In the majority of the specimens some or all of the openings to the outside become closed by the appearance of sphinc-

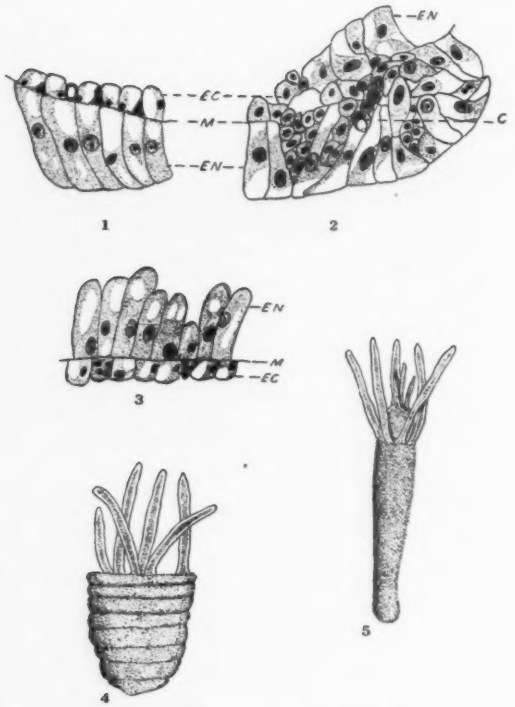
ters within a few minutes after turning. The hydra usually extends the free ends of its tentacles down along the sides of its body and continues to contract and expand them while holding them there. This procedure may pull the mouth of the animal open and cause the edges of the hypostome to be drawn back along the body. By alternate contractions and expansions of the tentacles the mouth is pulled backwards further and further until finally the base itself passes through the mouth. Finally, the contractions and expansions of the muscular elements easily force the basal disk back into its normal position, and then the hydra has recovered its original form.

The recovery of the normal orientation of the two body layers just described is a gross process, crudely comparable to the righting of a glove finger by re-turning it. The third class of turned hydras previously mentioned do not re-turn themselves in the accepted sense of the term, but nevertheless recover a normal organization by rearrangement of the cells constituting ectoderm and endoderm. This regeneration, as will be shown, is effected by migration of ectoderm and endoderm constituents in opposite directions through and beyond the mesoglea.

Sections of one hydra which was fixed two hours after turning showed the ectoderm and endoderm of the trunk in practically their normal positions. Other individuals required up to 8 hours or more to accomplish the same result. One specimen, a brown hydra, showed nematocysts in an exterior position twenty minutes after turning. Another, a green specimen, showed plainly that a considerable number of clear ectoderm cells had penetrated the mesoglea to a position on the outside within 30 minutes. Careful microscopic observations on the living turned *Hydra viridissima* have often disclosed the clear cells in the act of migrating to the outside between the green cells of the endoderm. Such cells become spherical, perhaps on account of freedom from pressure after they have passed through the green cell layer.

The stained sections of *Hydra vulgaris* and *H. oligactis* show the migration of cells very clearly. Figure 1 shows an arc of a cross-section of a specimen killed 10 minutes after the act of turning. Here the ectoderm is definitely on the inside, and no migration has as yet taken place. Figure 2, a section taken from an animal which had been turned 2 hours previous to fixing, shows the migration in progress. No definite mesoglea could be recognized in this animal except in certain isolated portions where the cells had not as yet commenced to move. Ectoderm and endoderm cells are found on both sides of a line drawn continuous with the remaining portions of the mesoglea. The large vacuolated cells are endoderm, while the smaller ectodermal cells are shown mostly without vacuoles.

PLATE I



EXPLANATION OF FIGURES

The upper portion of Figs. 1, 2, and 3 is the interior of the hydra. Here the ectoderm is shown in the internal position. *EN*, endoderm; *EC*, ectoderm; *M*, mesoglea.

FIG. 1. A portion of a cross-section of an everted hydra which was killed 10 minutes after turning.

FIG. 2. Portion of a cross-section of specimen after having remained everted for 2 hours. Some endoderm cells are shown here to have already reached the inside, while some still remain in their everted position. Masses of ectoderm cells are shown migrating through the section. Note the absence of mesoglea in most of the section. *C*, cnidoblast.

FIG. 3. A portion of a cross-section taken from a hydra 24 hours after eversion. Here the cell layers are shown in their normal condition after having completed the migration.

FIG. 4. Hydra immediately after turning, showing the arrangement of tentacles at the anterior end. The mouth of this specimen is not fully contracted.

FIG. 5. Hydra with bud after having been turned. Note the bud coming out of the mouth.

Figure 3 shows the condition of the cells of another specimen 24 hours after turning. The endoderm cells in this section have regained their interior position and proper alignment, and those of the ectoderm have assumed their normal arrangement on the exterior of the mesoglea.

The writer made sections of many specimens which were made too early or too late to show the migration convincingly. The obtaining of favorable material showing the migration at its height is more or less a matter of chance.

In the sponge, Wilson and Penney (1930) have shown that the cells migrate with somewhat amœboid movement, so it does not seem particularly striking that the cells in the hydra can wander. In fact, it has been shown by Hadzi (1909) that nematocysts migrate through the bodies of hydroids. Kepner and Barker (1924) have shown that the nematocysts of hydra can migrate from the enteron to the epidermis of worms of the genus *Microstoma*, which have ingested the hydra as food. In the face of these previous experiments, there is really nothing remarkable concerning the fact of the migration of the cells in the hydra, but they do not explain the method of migration. Personally, the writer feels that the cells migrate by almost imperceptible amœboid movements, although definite pseudopods have not been observed.

The fact of the filtering of ectodermal and endodermal elements through the mesoglea and through each other has been observed clearly in living and sectioned material, and definitely shows the older explanations of the changes occurring during regeneration to be in error. The writer can offer no plausible explanation for the *modus operandi* of the cell migration, which he has shown to occur, beyond the fact that similar phenomena have been observed in other instances.

The writer has no definite explanation for the rearrangements in the anterior end which lead to the reorientation of the tentacles and the hypostome. There appears, however, to be in this region a very complicated migration of cells which is very difficult to follow. The process seems to be initiated by the closing of the pores leading to the hollows of the tentacles, which, of course, are on the outside of the turned hydra.

Summary

1. Hydra may recover and continue to live after having been turned inside out.

2. The reactions to the turning may be classed in three groups: (*a*) "depression" and eventual dissolution; (*b*) re-turning; (*c*) regeneration through cell migration and without any re-turning.

3. Hydras that remain turned regain normality by the migration of the cells constituting the two layers of the body wall.

4. The migrating cells of each layer, individually or in groups, penetrate the mesoglea and filter between the cells of the opposing layer.

5. Hydras regenerated as described usually ingest food after 2 days from the time of turning.

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REACTIONS OF TWO HYDRAS, ONE OF WHICH HAS BEEN INGESTED BY THE OTHER

ANDREW L. PICKENS

It is well known that at times one hydra is swallowed by another, that the victim is retained for some time, and eventually regains its freedom, apparently none the worse for having been ingested. Few, however, have actually observed such an occurrence. One might linger around an aquarium for months, hoping for such an event, only to have his wait climaxed with disappointment. One might even place opposite ends of a small worm into the mouths of neighboring hydras with the hope of seeing the smaller ingested with the worm, only to see the weaker one drawn to the mouth of the other where the remainder of the worm is drawn from one enteron into the next while the losing hydra is permitted to drift away.

Several years ago, it was my pleasure to spend some time at work with hydras at the University of Virginia under the guidance of Dr. W. A. Kepner. Experiments were being made with beef-feeding, and the reactions of these cœlenterates to this unwonted food was indeed marked, and led to the discovery of a method by which one hydra could be made to ingest another, quite at the will of the experimenter.

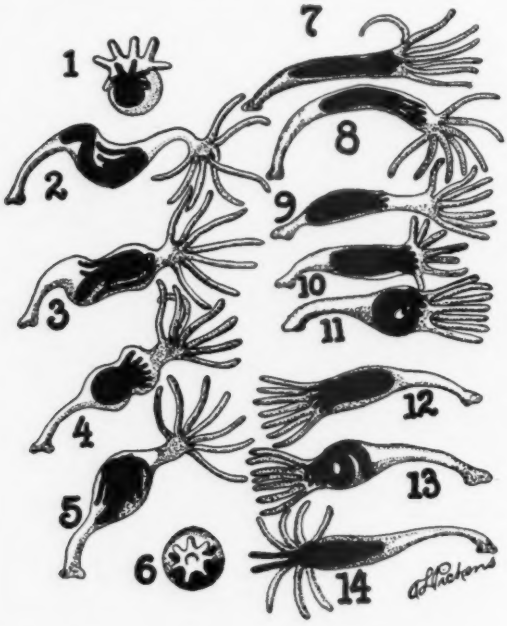
If the point of a dissecting needle is drawn several times through a piece of beef and then presented to the oral end of a hydra, it will receive the point of the instrument into the enteron, closing on the steel tightly with its entire interior length. The animal can then be lifted easily and transported to any part of the vessel. If, in this position, the animal is waved above the mouth of a larger hydra, the latter opens its mouth widely, whereupon the smaller animal may be readily thrust into the gaping enteron below. The reactions of such a pair are shown in the accompanying figures. The smaller was introduced into the gastrovascular cavity of the larger as the hour of 1:00 P.M. approached. The following reactions took place.

The larger animal contracted, assuming an almost globular shape (Fig. 1).

Both animals extended slowly, the contour of the outer fitting itself to that of the inner, the tentacles of which were all reversed. Motion on the part of the inner was followed by another contraction on the part of the outer, whereupon the inner also contracted (Fig. 2).

Another extension on the part of both occurred, and it could be seen that the prisoner's tentacles were being drawn forward. This motion of the interior hydra was again followed by contraction on the part of the outer hydra, immediately followed by a similar motion on the part of its captive (Fig. 3).

The outer animal extended once more. The inner one, as a result of the recent contraction was enabled to bring all its tentacles forward. Again the inner hydra contracted as did the outer (Fig. 4).



FIGS. 1–14. Two hydras, one of which has been ingested by the other. For description, see text.

Once again the prisoner had its tentacles reversed, and as a new extension occurred was seen to have been thrown across the body length of the outer hydra (Fig. 5).

At about 1:15 P.M. the outer hydra swung into a vertical position and again contracted. Observations were hindered by this for some time (Fig. 6).

At 1:30 P.M. the outer hydra was extended and lying prone, while the inner had the tentacles extended to the front. Its position would

have facilitated escape through the oral opening of the captor, but the latter contracted once more (Fig. 7).

At 1:45 the outer hydra had assumed the form of an arc in the top of which lay the body of the prisoner. A still longer extension of the outer hydra's body followed (Fig. 8).

The prisoner lost the ground gained, slipping further down and in than ever, touching the very bottom of the captor's enteron (Fig. 9).

An advance movement by the prisoner brought its fore regions into contact with the regions just within the hypostome of the captor (Fig. 10).

The prisoner assumed an almost circular form, which apparently helped it retain its position in the fore-end of the captor (Fig. 11).

At 1:51 the prisoner had straightened out again with the tentacles touching the inner portion of the captor's oral opening (Fig. 12).

The inner hydra, still just within the captor's hypostome, not only threw itself into a circle but actually coiled (Fig. 13).

By 2:28 the prisoner at last extended two of its tentacles through the captor's mouth into the open (Fig. 14). Liberty seemed imminent, when some movement on the part of the outer plunged the prisoner once more into the enteron of its captor. It appeared, however, to maintain its tentacles in the desirable forward-pointing position, and so was enabled to regain its lost ground more readily. At last at 2:51 the captor contracted in a different manner, for this time it evidently reversed the hypostome. Two forward movements were detected on the part of the captive, and it slipped out to freedom so rapidly that I was disappointed at my inability to make more lucid observations of what occurred. It had been a prisoner for approximately two hours.

The species used in this experiment was *Hydra viridissima.*

Additional experiments have shown the method to be practical for laboratory activities. It offers opportunity for tests as to whether or not two hydras repeatedly placed in this unusual relation would be able to sever the union more rapidly after a number of similar experiments had been undergone, as to the reception of macerated hydra flesh compared with living hydras, and other interesting problems.

OBSERVATIONS ON CERCARIÆUM LINTONI MILLER AND NORTHUP AND ITS METACERCARIAL DEVELOPMENT

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Introduction

This species was first recorded by Linton (1915), who found it in *Nassa* (= *Illynassa*) *obsoleta* (Say). He gave a brief description of the sporocyst and of the cercariæum, although he did not name the species. His account was confirmed and extended by Miller and Northup (1926), who named the larva in honor of Professor Linton. These authors made a twelve-month survey of the infestation of *N. obsoleta* with larval trematodes and found *Cercariæum lintoni* emerging at all periods of the year, with the greatest infestation in June and December. In a recent paper Africa (1930) described the excretory system of the cercariæum. He gave a figure of the flame cell pattern and a discussion of the mechanism by which the bladder and its accessory sphincter appear to function.

The present report contains the results of work done at the Marine Biological Laboratory during the summer months of 1930 and 1931. The study of marine cercariæ was suggested by Professor H. W. Stunkard, to whom I am indebted for suggestions and advice during the course of the work and in the preparation of this paper. Grateful acknowledgments are made also of assistance from Professor Edwin Linton, Professor P. W. Whiting, and Mr. Raymond M. Cable.

Material and Methods

Great numbers of snails, *Nassa obsoleta,* were obtained from Quisset Harbor and examined by the isolation method for specimens from which *Cercariæum lintoni* were emerging. These infected snails were placed in glass dishes where it was possible to keep them for days and even weeks. A daily change of water sufficed to maintain the snails in good condition and afforded a constant supply of these larvæ for experimental purposes. Morphological details were studied according to the methods discussed by Stunkard (1930). Compressed, unstained,

living cercariæ were observed for details of the flame cell pattern. Others, after staining with neutral red, were used in the study of the different glands. Permanent mounts were made also of specimens fixed and stained by various methods.

Experiments were carried on to determine the effects of changes in environmental conditions, and others were performed in an attempt to elucidate the life history and secure the adult form of the species. Certain features of the cercariæ, (1) the presence of a stylet, (2) presence of cephalic, probably penetration glands, (3) failure to encyst on the walls of the container, and (4) great endurance of changes in environmental factors, appeared to be of significance for developmental studies and suggested that they encyst in a second intermediate host. Accordingly, various invertebrates were exposed to active cercariæ to find out whether penetration and encystment would occur.

TABLE I

Measurements of Cercariæum lintoni. All measurements are given in millimeters, and each is the average of several measurements. The figures of Linton and Shaw are for living specimens, while those of Miller and Northup are for fixed specimens.

	Linton	Miller and Northup	Shaw
Length	.327	.23	.355
Width, anterior	.06		
" middle	.123	.084	.118
" posterior	.06		
Oral sucker (diameter)	.043		.05
Ventral sucker (diameter)	.0623		.07
Excretory vesicle, length			.07
" " width			.045
Pharynx, length			.025
" width			.022
Stylet, length	.02		.0186

MORPHOLOGY OF THE LARVÆ

Sporocyst Stage

The appearance, form, and size of the sporocysts were reported by Linton. He found them to average 0.645 mm. in length, 0.272 mm. in width, and to contain 2 to 40 cercariæ per sporocyst. My observations are in substantial agreement. There are about 18 to 20 cercariæ in a medium-sized sporocyst and their arrangement is shown in Fig. 3. Encystment of the cercariæ within the sporocyst was never observed.

Cercariæum

As noted by Linton, the cercariæ manifest much difference of form in extension and contraction. They are elongate forms, usually ovate, with the greatest width at the region of the ventral sucker or just posterior to it. Usually the anterior end narrows gradually and the posterior tip is slightly angular. A specimen full extended measured 0.83 mm. in length and 0.055 mm. in greatest width, while in a contracted condition it was only 0.25 mm. long. Table I gives measurements of the larva and certain of its structures as recorded by different authors.

The entire surface of the body is covered with minute spines; those of the anterior region are somewhat larger than those farther posteriad. They are set in rows, approximately 250 in number, arranged in alternate fashion as indicated in Fig. 9. Linton reported that the anterior spine (stylet) is not easily seen in mounted specimens, while Miller and

PLATE I

Explanation of Abbreviations in Figures

c—cercariæum
cp—cirrus pouch
dg—ducts of cephalic glands
e—esophagus
ec—encysted cercariæum
ed—ejaculatory duct
es—excretory sphincter
et—lateral excretory tubule
ev—excretory vesicle
g—cephalic gland
gl—gland posterior to ventral sucker
gp—genital pore
ic—immature cercariæum
icw—inner cystic wall
inc—intestinal cecum
m—metraterm
o—ovary
ocw—outer cystic wall
og—opening of cephalic gland duct
ogl—opening of duct of gland posterior to ventral sucker
os—oral sucker
ov—oviduct
p—pharynx
pp—prepharynx
s—stylet
sr—seminal receptacle
sv—seminal vesicle
t—testis
u—uterus
vd—vas deferens
vs—ventral sucker

Explanation of Figures

Fig. 1. *Cercariæum lintoni* Miller and Northup, ventral view.

Fig. 2. Anterior portion of *C. lintoni* showing the relationship of the stylet and the ducts of the cephalic glands when the stylet is drawn within the oral sucker.

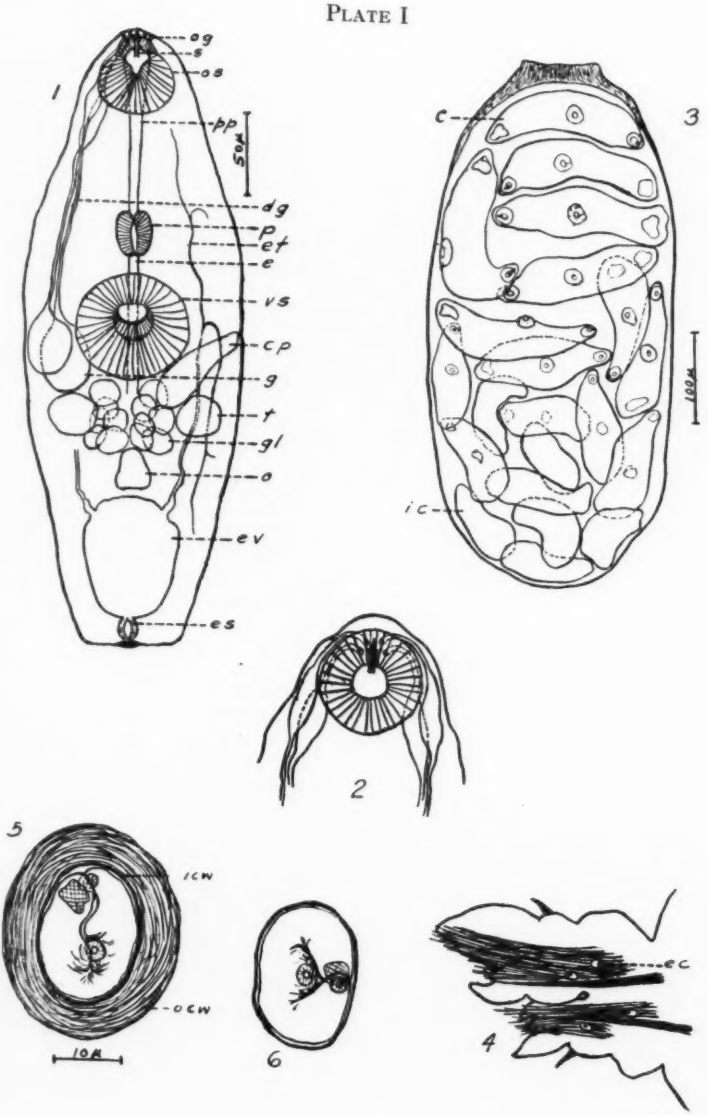
Fig. 3. Diagram of a sporocyst of *C. lintoni.* Note the relative positions of mature and immature cercariæ.

Fig. 4. Diagrammatic drawing of a parapodium of *Nereis virens* Sars., showing the characteristic location of cysts.

Fig. 5. Camera lucida drawing of a ten-day-old cyst of *C. lintoni.* Note the two layers of the cyst wall.

Fig. 6. Camera lucida drawing of the same ten-day-old (Fig. 5) cyst with the cercariæum in a different position.

PLATE I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Northup merely sketched it in their figure without mentioning it in their description. It is prominent in all active, living specimens and is shown, much as Africa (1930) figured it, in Figs. 1 and 2.

All accounts of species agree as to the nature and location of the prepharynx and pharynx, but there is considerable disagreement as to the intestine. Linton stated that, "intestinal rami were not distinctly shown" while Miller and Northup reported "very narrow intestinal ceca, traceable only in serial sections, reaching almost to the excretory vesicle." Miller and Northup, and Africa, figure a bifurcation of the short esophagus at the anterior margin of the ventral sucker and slender intestinal ceca which extend caudally. The writer was unable to distinguish such intestinal ceca, and study of later stages indicates that they probably do not exist.

The description of the excretory system, begun by Linton and emended by Miller and Northup, was completed by Africa. He gave the excretory formula as $2[(2+2)+(2+2)]=16$ flame cells, and figured the flame cells with connecting tubules. My observations are in full accord and verify his conception of certain connecting tubules of which he was not positive.

Rudiments of the reproductive organs were observed by Linton and also by Miller and Northup. Linton's observation concerning the testes is confirmed but the structures he identified as the rudimentary ovary and the "beginning of the uterus" have been shown in the present study to be the beginnings of the cirrus sac and the ovary respectively. He suggested that certain granular masses might be the "beginnings of diffuse vitellaria," but this point is still uncertain.

The two pairs of cephalic glands shown in Fig. 1 have been described by previous workers. But in addition, by intra-vitam staining with neutral red, I have been able to recognize six pairs of glands in the region just posterior to the ventral sucker.

Experimental Studies

Environmental Effects

The effects of various dilutions of sea water on the activity and longevity of these cercariæ were reported (Stunkard and Shaw, 1931). Specimens placed in 50 per cent and 25 per cent sea water remained alive as long or even a few hours longer than those in undiluted sea water. Room temperature was used in all of these experiments. The cercariæ lived, under these conditions, for 36 to 70 hours.

The cercariæ were much more resistant than those of other species, such as *Cercaria quissetensis* Miller and Northup (from the same host), on exposure to low temperatures. A dish containing hundreds of

recently emerged specimens was placed at a temperature of 4° to 6° C. The early reaction was an increase in activity, but an hour later they were less active and there was a gradual decrease of activity during the remainder of the lifetime. By the eighth day a few had died, by the tenth day the majority were dead, and only a few showed slight activity on the twelfth day. The same experiment with *Cercaria quissetensis* showed that species to be much less resistant; each cercaria had ceased all activity by the end of the fourth day.

Infection Experiments

Since the cercariæ are tailless, they are unable to swim and an intermediate host must be present in the immediate vicinity of the snail from which they emerge. Miller and Northup described their activity as, "inch-worm locomotion . . . by successive attachment of posterior end of body, modified by invagination into an adhesive disc, then extension of body and attachment by ventral sucker." Very rarely one was found that had encysted on the side of the dish, but usually they showed gradual decrease in activity unto the point of death.

Nereis virens Sars. was successfully infected in the first attempt and gave positive results in all later experiments. The worm was placed in a bowl of sea water containing several hundred recently emerged cercariæ. After an exposure of from two to five hours it was removed to a specimen bowl, which contained well washed sand one-half inch deep. The bowl was then covered with a piece of scrim cloth held in place by a rubber band and placed under a slowly running stream of sea water. In this manner the infected worm could be kept for several weeks. It was interesting to note that cysts were never found in any part of the host other than the parapodia. Their typical location in the parapodium is indicated in Fig. 4; no doubt the rich blood supply in this region affords especially favorable conditions for development of the metacercariæ. At frequent intervals, parapodia were clipped off and the cysts dissected out and studied. The metacercariæ were removed by slightly crushing, or dissecting the cysts, and studied by the same methods used for the cercariæ.

Various invertebrates, including other annelids (*Hydroides* sp., *Lumbrinereis hebes, Scoloplos robustus,* and *Arabella opalina*) [1] and the flatworm, *Bdelloura candida,* were used in similar studies. These species were selected since they may occur in the same habitat as *Nassa obsoleta* and conceivably might serve as hosts of the parasite. In each instance the cercariæ crawled over the surface of the exposed animal and a few penetrated the body wall and encysted. Such cercariæ, when

[1] The author is endebted to Dr. L. P. Sayles, Department of Biology, College of City of New York, for identification of the last three forms.

removed from their cysts, did not manifest the activity and developmental changes that characterize metacercariæ dissected from cysts in *Nereis virens*. In no other host did development occur; on the contrary the larvæ died and the cysts became brown and opaque, according to numerous examinations.

To observe the method of penetration, parapodia were cut from non-infected *Nereis* and placed in Syracuse watch glasses with many active cercariæ. In a few moments the cercariæ were crawling over the parapodia. An hour later several were in very much contracted form and seemed to be inactive but attached to the parapodia. A short time later some of these worms, with anterior end against the parapodium, showed actual "pecking" and boring movements. At the end of two hours some had the anterior end well buried in the parapodium with the caudal end showing great muscular activity. At this time one was observed to be buried in the tissue with only the extreme posterior tip free. One hour later, examination with the binocular showed some within the parapodia and with walls around them. Since the parapodia began to show signs of disintegration at this point the study was not carried farther. It seems quite evident that the cercariæ actually bore their way into the fleshy parapodia by means of the stylet, and presumably the secretion of certain glands, notably the cephalic glands, aids in the process.

Nereis virens has proved to be an extraordinarily convenient host for such a study. As stated above, the experimental method of infestation was very simple since all that was necessary was to isolate a worm in a dish with hundreds of active cercariæ for two to five hours. Each time such an exposure was made the majority of parapodia, examined later, contained from one to six cysts—the results of the penetration and encystment of as many cercariæ. The infected *Nereis* could be kept alive for weeks and did not seem to suffer from the periodic clipping of its parapodia.

Development of the Metacercaria in Nereis

The development of the metacercaria within its cysts is typical, since there is a gradual reduction of larval structures with concomitant growth and differentiation of the organs of the adult stage, which is yet unknown. The transformation of the larva from cercaria to metacercaria is shown in Figs. 6, 7, 8, 10, and 11.

The Cyst

The cysts may be readily observed in the parapodia with a binocular microscope. The wall of the cyst is quite transparent in early stages and never becomes truly opaque, even in later stages, as long as the meta-

cercaria is alive. In older stages the cyst wall consists of two coats, as shown in Fig. 5. The inner coat, which is formed from glandular material of the larva, is thin, clear, and easily ruptured. Around it there develops a thick coat of connective tissue, produced as a reaction of the surrounding tissues of the parapodium, which is tough and difficult to break. Pressure on the cyst causes the contained worm to squirm and it is possible to observe certain features very clearly. The excretory vesicle is especially prominent, as it is filled with concretions that make it appear dark. The cysts increase in size as the metacercariæ develop; Table II summarizes measurements made of cysts of various ages.

Loss of Larval Structures

The large cephalic, or penetration glands are resorbed and have disappeared entirely by the sixth day. The stylet is clearly visible and

TABLE II

Measurements of cysts of Cercariæum lintoni M. and N. which had been dissected out of Nereis. Each figure is the average of eight or more measurements made of forms in an uncovered drop of water.

Age	Length	Width
days	*mm.*	*mm.*
4	.15	.13
13	.21	.155
26	.246	.202
36	.258	.191
45	.26	.225

apparently unchanged during the first ten days of the encysted stage. By the thirteenth day it is reduced in size and the form is somewhat modified as the tip is very blunt. In the cercarial stage the stylet measures 0.0186 mm. in length and 0.033 mm. in width. In the thirteen-day-old metacercaria it measures 0.0156 by 0.0022 mm.; in the twenty-two-day-old 0.0066 by 0.002 mm.; and in the twenty-six-day-old the stylet is lacking completely.

Development of Adult Characters

Certain structures, e.g. the details of the excretory system, are established in the cercarial stage and seem to remain constant throughout development. But the digestive system and the reproductive organs become more developed, or at least more evident, as the metacercariæ increase in age and size.

As discussed previously, the exact nature of the digestive system in the cercarial stage is doubtful. The prepharynx is long with the pharynx just anterior to the ventral sucker. The first positive observations of the post-pharyngeal region of this system obtained by the writer were in the study of a living ten-day-old metacercaria, in which it was possible to see a single tube extending posteriorly. Liquid, with suspended granules, was being forced back and forth in this tube. In the study of metacercariæ 30 days old and older, it was easy to see the exact nature of the entire system (see Fig. 11) in both living and mounted specimens. The esophagus extends posteriorly, dorsal to the gonads, and in the region posterior to the cirrus pouch it gives rise to two large bulb-like intestinal ceca.

The reproductive organs, as they appear in metacercariæ 35 to 49 days old, are shown in Fig. 11. The testes are located exactly as Linton described them in the cercariæum. The vasa deferentia are short, slightly coiled tubules leading to the cirrus pouch, in which they unite to form a seminal vesicle. From the vesicle an ejaculatory duct traverses the cirrus sac and the genital pore is situated on the left margin of the body at the level of the aperture of the acetabulum. The ovary is situated just anterior to the excretory vesicle, as reported by Miller and Northup. The oviduct is short and communicates with a seminal receptacle on the right side of the body, while the uterus extends as a long tortuous canal to the metraterm which is parallel to the cirrus sac and opens at the genital pore. The vitellaria were not located with certainty.

Measurements of metacercariæ of various ages (dissected from their cysts) are given in Table III. These measurements were used in part in constructing Figs. 10 and 11. Comparison with Table I shows the extent of development.

Adult Host

Only one attempt was made to determine the host of the adult trematode and this experiment was unsuccessful. The host of the

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EXPLANATION OF PLATE II

FIG. 7. A diagrammatic sketch of a ten-day-old metacercaria emerging from its cyst which has been crushed by slight pressure.

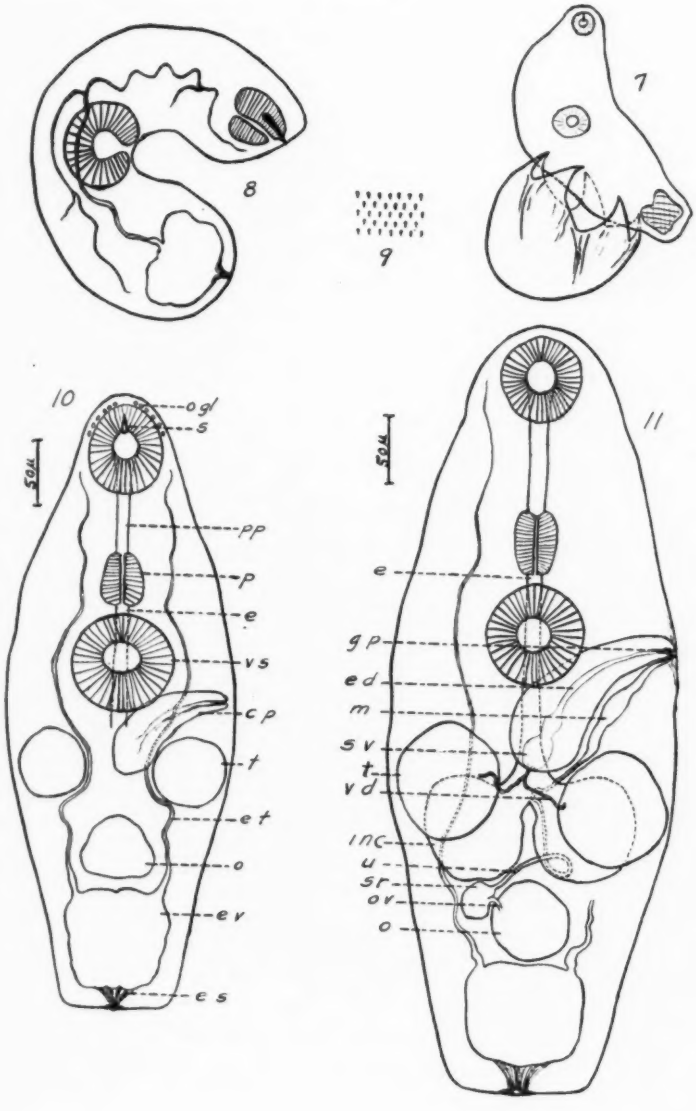
FIG. 8. Lateral view of a two-day-old metacercaria of *Cercariæum lintoni*, showing the relationship of the most prominent structures of that stage.

FIG. 9. Diagram to show the plan of arrangement of the spines which cover the surface of the body of *C. lintoni* and its metacercaria.

FIG. 10. Diagrammatic drawing of 18-day-old metacercaria of *C. lintoni*. Ventral view.

FIG. 11. Diagrammatic drawing of 45-day-old metacercaria of *C. lintoni*. Ventral view.

PLATE II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adult is, presumably, a fish which eats *Nereis*. Since the eel and *Nereis* have been observed in the same habitat in the Woods Hole region, since eels are believed to eat clam worms, and since the eel is a hardy animal which will withstand laboratory conditions, this species was selected for the experiment. On August 7, 1931 two infested *Nereis virens* (one containing 45- and the other 38-day-old metacercariæ) were fed to an eel, *Anguilla chrisypa* Raf. The eel died three weeks later but no trematodes were found.

Discussion

When it was discovered that *Cercariæum lintoni* would develop in *Nereis virens*, it appeared probable that advanced metacercarial stages

Table III

Measurements of metacercariæ of Cercariæum lintoni M. and N. All measurements are in millimeters; all measurements made from living specimens.

	13 days old	18 days old	27 days old	45 days old
Length	.42	.46	.47	.575
Width	.15	.1725	.24	.215
Oral sucker, length	.075	.07	.07	.065
" " width	.06	.06	.0625	.06
Ventral sucker, length	.07	.075	.075	.075
" " width	.085	.08	.075	.075
Pharynx, length	.035	.04	.042	.0484
" width	.032	.035	.038	.0374
Testis, length	.065	.06	.065	.08
" width	.06	.06	.07	.085
Ovary, length	.05	.05	.055	.06
" width	.06	.06	.06	.06
Excretory vesicle, length	.075	.075	.07	.075
" " width	.07	.08	.09	.09
Stylet, length	.0156	.0143	.0066	—
" width	.0022	.0022	.002	—

would be sufficiently mature so that comparison of them with descriptions of marine trematodes might suggest the adult form. It was thought that at least the family to which they belong could be determined. This hope has not, however, been realized. Examination of the papers by Looss (1894), Lühe (1909), Odhner (1911), Linton (1898, 1899, 1904, 1907, 1910), Manter (1926, 1931), and Fuhrmann (1928) has failed to disclose a trematode that could be considered as the adult form of *C. lintoni*.

Palombi (1930) worked out the life history of a tailless cercaria at Naples and found that it developed into *Diphterostomum brusinæ* (Stossich), a member of the family Zoogonidæ. He compared the cercaria of

D. brusinæ with *Cercariæum lintoni* and concluded that they were closely related species. He stated: "*Cercariæum lintoni* Miller e Northup parassita die *Nassa obsoleta* (Say), presenta sensibili affinitá con la cercariæ di *Diphterostomum brusinæ* Stoss. parassita di *Nassa mutabilis* L., *N. reticulata* L. e *Natica Poliana* Delle Chiaie. Non e improbabile che essa rappresenti la forma larvale die una specie della famiglia *Zoogonidæ* Odhner." Table IV gives a detailed comparison of the two

TABLE IV

Comparison of the cercariæum of Diphterostomum brusinæ Stossich and Cercariæum lintoni Miller and Northup

	Cercariæum of *D. brusinæ* Stoss.	*Cercariæum lintoni* M. and N.
Length	.23 mm.	.32 mm.
Width	.10 mm.	.102 mm.
Size of ventral sucker	Considerably larger than oral sucker.	Slightly larger than oral sucker.
Position of ventral sucker	Just posterior to middle region.	In middle region.
Lateral view of v. sucker	Shows two peculiar "prominences."	Without peculiar prominences (Fig. 8).
Stylet	Present.	Present.
Pre-pharynx	None.	Long.
Pharynx	Just posterior to oral sucker.	Just anterior to ventral sucker.
Esophagus	Long.	Rather long.
Intestinal ceca	Slender and short.	Short, bulb-like.
Testes	Just posterior to ventral sucker.	Just posterior to ventral sucker.
Genital pore	Median, slightly anterior.	Lateral, left, just posterior to ventral sucker.
Sporocyst	In *Nassa mutabilis*.	In *Nassa obsoleta*.
Free living stage	None.	Emerges from snail; active for hours.
Encystment	In snail host; in or out of sporocyst.	In parapodia of *Nereis virens*.
Mode of entrance into next host after snail	Eaten by fish.	Penetrate parapodia of *Nereis virens*.

species and indicates that they are not as closely related as Palombi believed.

Palombi suggested that *Cercariæum lintoni* may be the cercarial form of a species of *Deretrema* Linton, Family *Zoogonidæ*. The morphological characters of the metacercaria show, however, that the species does not belong to the genus *Deretrema*. In the arrangement of reproductive organs it agrees with members of the family *Zoogonidæ*, but

the digestive system is very different. The digestive system of the metacercaria resembles that of *Saccocoelium* Looss (Family *Haploporidæ*), but the reproductive organs do not correspond to those of the *Haploporidæ*. It is, accordingly, impossible to assign *C. lintoni* to any family as they are at present characterized, and determination of the systematic position of the species must await further knowledge.

Summary

The description of *Cercariæum lintoni* Miller and Northup as given by earlier workers is supplemented and emended in the light of knowledge obtained by comparing the cercariæum with its metacercarial stage, developed in experimental animals. The true nature of the digestive and reproductive systems is made clear.

Cercariæum lintoni penetrated the parapodia of *Nereis virens*, encysted, and developed into the metacercarial stage. Although the cercaria would penetrate and encyst in other invertebrates, the clam worm was the only form in which development was secured. *Nereis virens* appears to be a natural host. The metacercaria develops adult features as the larval characters disappear.

It is difficult to determine the relationship of this species to other trematode groups; the digestive system is similar to that of the species of the family *Haploporidæ*, but the arrangement of the reproductive organs is very different and resembles that of the *Zoogonidæ*. Palombi's suggestions concerning the relations of the species are discussed.

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